Role of Growth Factors and Extracellular Matrix Regulators in Airway Remodeling in COPD

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Role of Growth Factors and Extracellular Matrix Regulators in Airway Remodeling in COPD

De rol van groeifactoren en extracellulaire matrix regulatoren in luchtweg remodeling in COPD

Proefschrift

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Voor Simone

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General introduction and aims of the study

General Introduction

1.1 Definitions of COPD

Chronic Obstructive Pulmonary Disease (COPD) is a chronic inflammatory disorder of the lungs, becoming a global health problem with increasing morbidity and mortality (1). Recent observations indicate that COPD is the fourth cause of mortality in the USA and it is projected to be the fifth burden of morbidity world-wide in the year 2020 according to a consensus report published by the World Health Organisation (2).

COPD is characterized by a slow progression of airflow limitation, which is nearly irreversible. Recently, the Global Initiative on Obstructive Lung Disease (GOLD) has formulated an official definition; "A disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lungs to noxious particles and gases" (2). One of the major determining factors is tobacco smoking, but it remains to be investigated to what extent other factors such as environmental and occupational exposures and genetic factors can contribute to the disease. Surprisingly, only 10-20 percent of all smokers develop COPD (1, 2).

Diagnosis of COPD should be considered in patients with symptoms of cough, sputum production and abnormal shortness of breath and a presumed history of exposure to risk factors for the disease (1, 2). The diagnosis is confirmed by spirometry with a post-bronchodilator forced expiratory volume in one second (FEV_1) < 80% of predicted value and in combination with an FEV_1/FVC (forced vital capacity) < 70% of predicted. The above lung function criteria are used in classifying the severity of the disease as stage I (mild COPD) followed by stage II (moderate COPD) with FEV_1 values 30% to 80% of predicted and stage III (severe COPD) with FEV_1 values < 30% of predicted (2). Figure 1.1 illustrates the effects of smoking on the annual decline in lung function (FEV_1) of susceptible and non-susceptible smokers and also depicts the beneficial effect of smoking cessation. These data originate from a large epidemiological study in Britain from 1977 (3), and have subsequently been confirmed in more recent (4, 5).

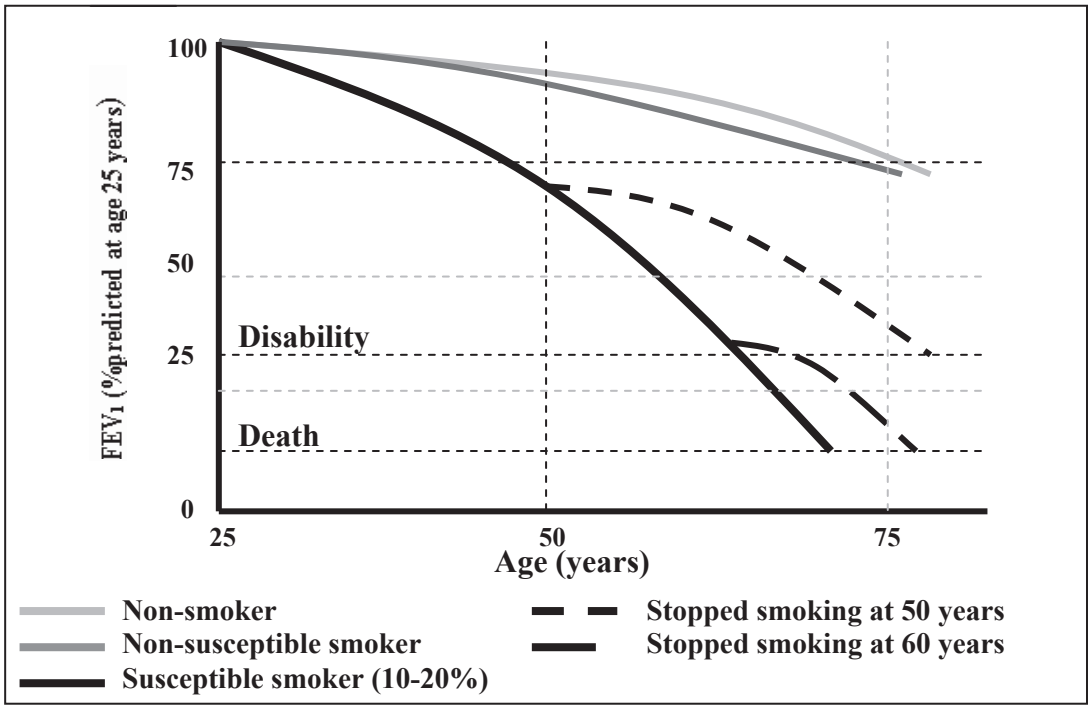

Figure 1.1 **Annual decline in forced expiratory volume in 1 second (FEV_1 values are given in % predicted).** Susceptible smokers show an accelerated decline in lung function as compared to non-smokers and non-susceptible smokers. The beneficial effects of smoking cessation are also depicted. Adapted from Refs. (1, 3).

1.2 Structure of airways and lung parenchyma

The respiratory system is commonly divided in two separate parts: the conducting airways consisting of trachea, bronchi, bronchioles, terminal bronchioles and the respiratory part defined as respiratory bronchioles, alveolar ducts and terminal alveoli (6). The entire branching pulmonary tree consists of roughly 20 to 25 generations and a branch of the pulmonary artery accompanies each conducting airway (6). Conveniently the different anatomical sites are divided as "central" lung tissue, representing the larger conducting airways and "peripheral" lung tissue, which includes terminal and respiratory bronchioles, and alveoli. Airways with a diameter of 2 mm or less are conveniently defined as small airways, which are considered as the most important contributors to the airflow resistance and are involved in the accelerated decline of FEV_1 in COPD (7-11). Figure 1.2 shows the important structural features of "central" and "peripheral" lung tissues in case of non-symptomatic smokers (A and B) and COPD (C and D) subjects, respectively. Conducting airways consists of an epithelial layer, its basement membrane, and the

lamina propria, that consist predominantly of connective tissue and small vasculature, together forming the airway mucosa (6). The bronchial epithelium, covered with secreted mucus, protects the outer layers from first contact with the air or pathogens in the lumen. Different cell types are found in the epithelial layer, the cubical shaped ciliated cells, the secretory cells such as goblet and Clara cells which play a role in the production of mucus and the smaller basal cells which are though to be the epithelial stem cells (12). In the submucosa of the central airways irregular shaped patches of submucosal secretory glands and airways smooth muscle are found. In the adventitia of the larger airways predominately cartilage, supplying bronchial vasculature and connective tissue are observed, with a slow transition into the more peripheral areas of the lungs (13).

In peripheral tissue terminal and respiratory bronchioles as well as alveolar ducts and alveoli with accompanying arteries are present (13). Veins of several sizes are found predominantly in interstitial septa, which are rich in extracellular matrix fibres such as collagens. While branching bronchioles gradually lose their coating of secretory glands, cartilage and finally also their ASM layer (6, 13). The alveolar walls are covered with flattened respiratory epithelial cells, alveolar type I cells, which are responsible for most of the gas exchange with capillaries in close proximity, and with more cubical shaped cells, alveolar type II cells, that are progenitor for the latter cells (14-16). Furthermore a scattered population of immune cells, predominantly a low number of alveolar macrophages, T-lymphocytes and granulocytes, is found (17).

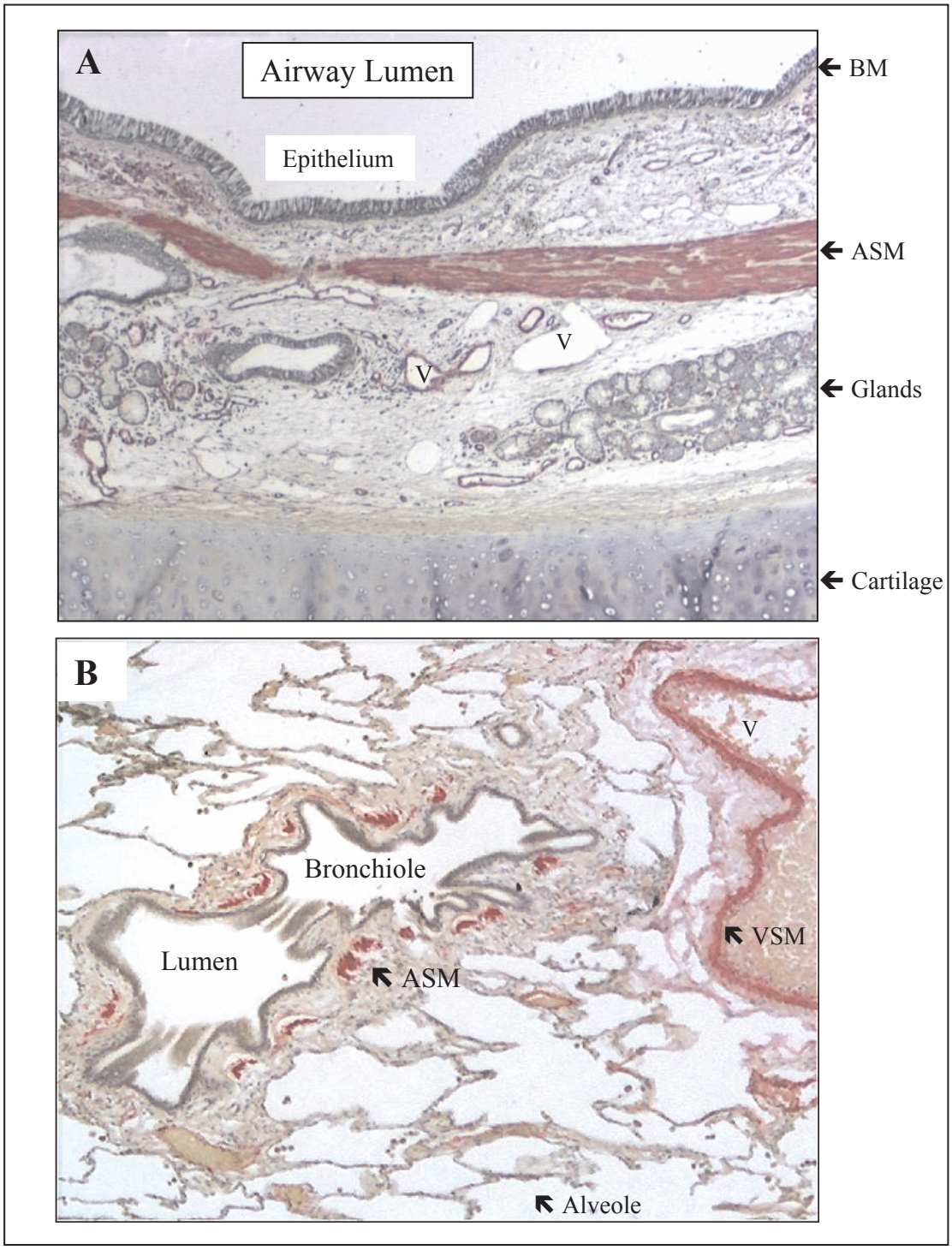

Figure 1.2 **The important structural features of "central" and "peripheral" lung tissues in case of non-symptomatic smokers (A and B) and COPD (C and D) subjects, respectively.** Panel A depicts the main central airway structures with the bronchial epithelium, basement membrane (BM), the arterioles, capillaries, and venuoles embedded in the subepithelial layer (V) as well as airway smooth muscle (ASM) and subepithelial glands (Glands) and cartilage of the airway wall. In B a small bronchiole with its epithelium lining the lumen and ASM is embedded in the surrounding alveoli. An accompanying vessel (V) is present surrounded by vascular smooth muscle (VSM).

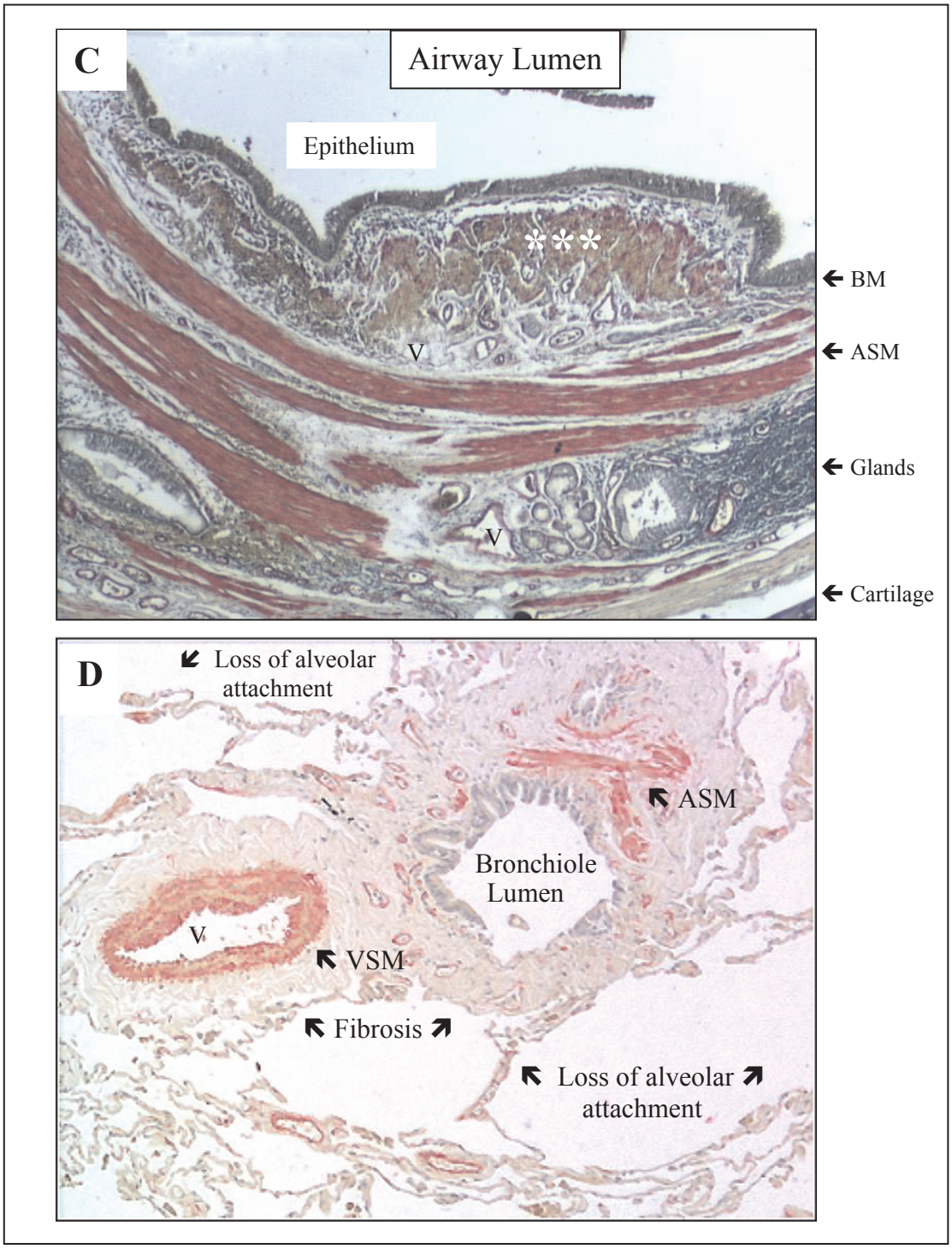

Figure 1.2 **(continued)** In panel C can be observed that subepithelial fibrosis (***) is present as well as an increased airway smooth muscle mass in COPD as compared to non-symptomatic subjects. Panel D shows important peripheral features of COPD, peribronchial and perivascular fibrosis, vascular wall thickening and emphysema with enlarged air spaces and loss of alveolar attachment. All slides are stained with α-smooth muscle actin (red color).

1.3 Pathology and pathogenesis of COPD

COPD consists of three distinct pathological conditions under one umbrella (Figure 1.3). These are chronic bronchitis with productive cough of more than three months and mucus hypersecretion, small airway disease with chronic obstruction and inflammation of smaller airways, and emphysema with enlargement of air spaces, destruction of lung parenchyma, loss of lung elasticity that can in turn cause collapse of respiratory airways (18).

Excessive tobacco smoking is the main cause in the pathogenesis of COPD, which can be explained by observations that inflammatory reactions are present in the entire tracheo-bronchial tree of non-obstructive smokers. Studies in the central airways indicate that the inflammatory infiltrate predominantly consists of cytotoxic $CD8^{+ve}$ T-lymphocytes, neutrophils and macrophages in the airway wall and neutrophils in the bronchial lumen (19, 20). Moreover, in the small airways and parenchyma of young non-obstructive smokers already an inflammatory cellular infiltrate is found without any structural changes, which could pinpoint towards initial stages in the pathogenesis of the disease (11). In COPD patients the cellular infiltrate is further increased in the small airways consisting predominantly of $CD8^{+ve}$ T-lymphocytes, neutrophils, macrophages as well as mast cells (21-23).

Cellular and structural changes in smokers with or without COPD are summarized in Table 1.1. Smokers with airflow limitation show changes in peripheral airways including inflammation, fibrosis, mucus plugging and airway smooth muscle hypertrophy (21, 22, 24-27). These factors cause deformation and narrowing of the airways and together with destruction of alveolar walls, could lead to airflow limitation. Less attention has been focused on central airways in COPD. The airway wall showed a further increase in the number of macrophages and T-lymphocytes and the airway lumen an elevated influx of neutrophils. Furthermore, changes in central airway dimensions with increased submucosal fibrosis and airway smooth muscle mass are observed in COPD patients compared to non-symptomatic subjects (8, 28).

The cellular and molecular mechanisms, which may explain the slow progression of airflow limitation, however, are not entirely clear. The currently well accepted protease-antiprotease hypothesis states that as a result of this smoke-induced ongoing inflammatory process, the connective tissue of the lungs is degraded by a relative excess of inflammatory-cell derived proteases such as neutrophil and

macrophage elastases and a relative depletion of antiproteolytic defences like α_1-antitrypsin or secretory leukocyte proteinase inhibitor (SLPI), (29, 30). Moreover, an unbalanced expression and release of anti- and pro-inflammatory cytokines or growth factors may play an important role.

Although definitive progress has been made in the understanding of the disease and several drugs that can diminish symptoms in COPD patients like corticosteroids, bronchodilator agents or anti-inflammatory compounds have been found, no drugs are available at present that can reduce the progression of the disease (1). The only effective therapeutic intervention currently available is smoking cessation, but the effects only account for the diminishing of future damage since the disease state is poorly reversible (31).

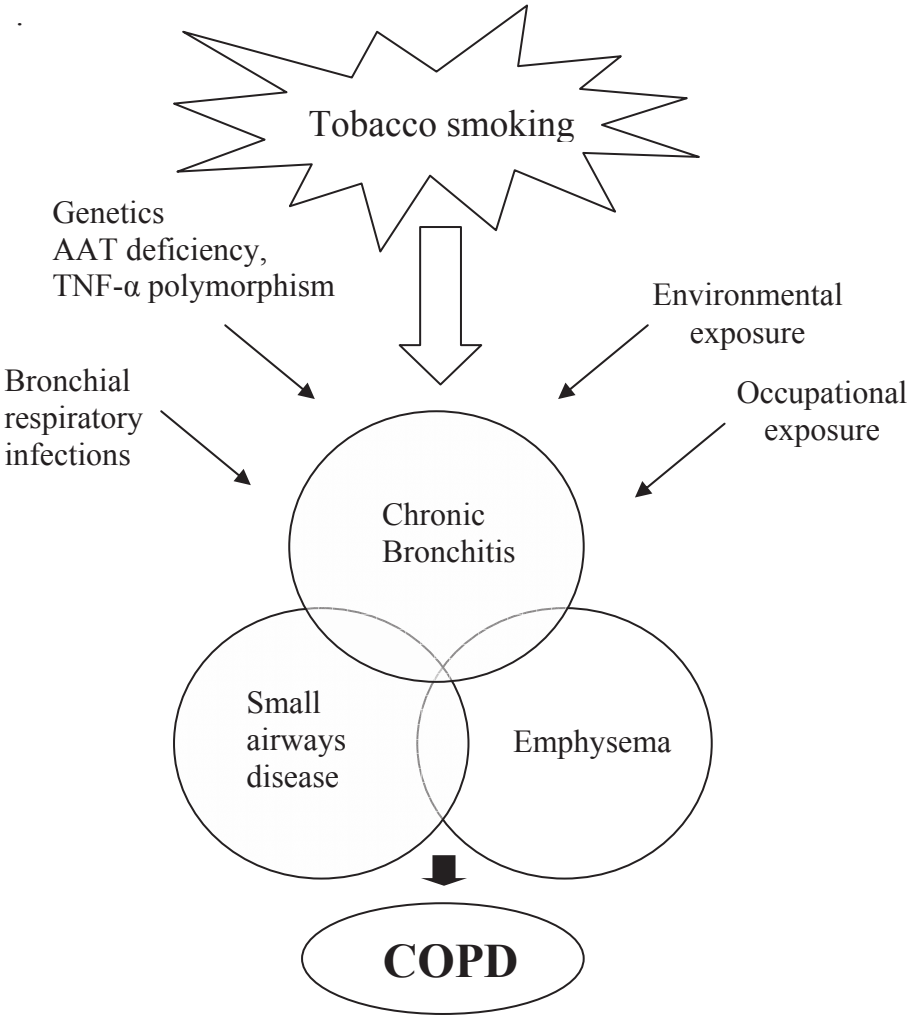

Figure 1.3 **Risk factors for COPD.** Among several other possible factors involved in the development of COPD, excessive smoking is considered as the main risk factor for the disease. COPD comprises of three distinct pathological conditions under one umbrella, chronic bronchitis, small airway disease and emphysema. AAT = α_1-antitrypsin, TNF-α = tumor necrosis factor α.

TABLE 1.1 CELLULAR AND STUCTURAL CHANGES PRESENT IN THE LUNGS OF NON-SYMPTOMATIC SMOKERS AND OF SMOKERS WITH ESTABLISHED COPD

	Non-symptomatic smokers	Smokers with established COPD
Central airways		
Wall	T-lymphocytes Macrophages	Further increase in macrophages, $CD8^{+ve}$ T-lymphocytes Neutrophils in severe disease
Lumen	Neutrophils	Neutrophils
Peripheral airways	Mononuclear cells Clusters of macrophages in the respiratory bronchioles	Goblet cell metaplasia and mucus plugging Smooth muscle hypertrophy Fibrosis Macrophages, mast cells, neutrophils in severe disease $CD8^{+ve}$ T-lymphocytes
Parenchyma	No destruction No fibrosis	Inflammation $CD8^{+ve}$ T-lymphocytes Destruction centriacinar and panacinar emphysema Fibrosis
Pulmonary arteries	Intimal thickening	Endothelial dysfunction Intimal thickening Medial thickening Adventitial inflammation $CD8^{+ve}$ T-lymphocytes

Based on References (8, 20, 23, 32-34).

1.4 Tobacco-induced injury and repair

It is now well established that particles from the smoke can cause damage to the airways in particular to the epithelial lining (35). Loss of epithelium induces repair processes, which consists of many steps and involves many factors (36). The role of damage in the in COPD is less clear, since it could originate from direct effects of smoking and/or from the subsequent chronic inflammation. Yet, both non-symptomatic smokers and established COPD patients show signs of damage and repair to the epithelial surface in the form of denuded epithelial lining and also squamous metaplasia (8).

Normal wound healing

The processes of normal and abnormal wound healing as a response to injury have been studied thoroughly (37-41). Figure 1.4 schematically summarizes the important cellular events in normal wound healing (41).

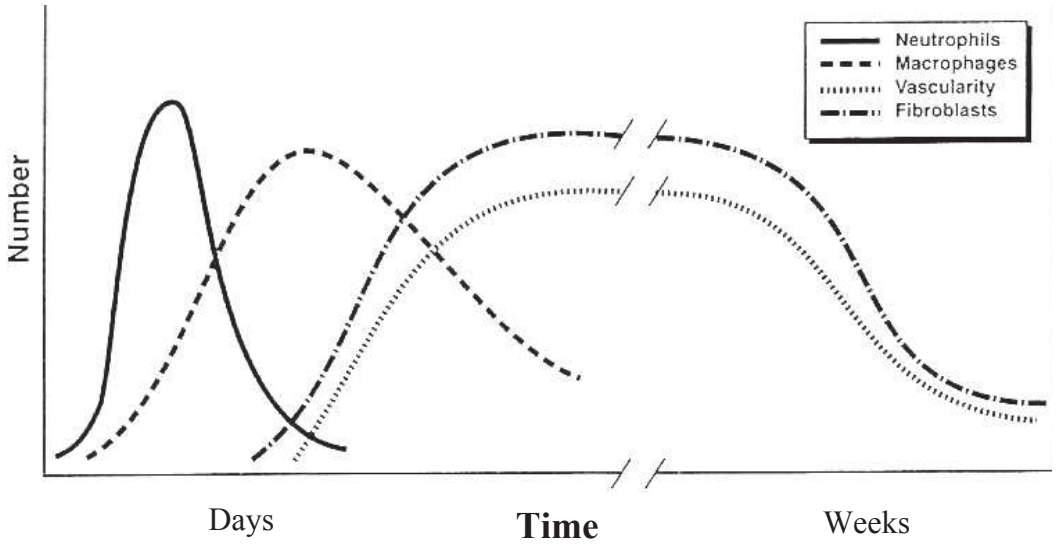

Figure 1.4 **Summary of the important cellular events in normal wound healing.** During tissue repair several cell types undergo a rapid and transient increase in number that eventually drop to negligible levels by the time the wounded area reaches maturation. Normally, redundant inflammatory and structural cells undergo apoptosis and the wounded area ends up comparatively acellular and avascular. Adapted from Ref. (41).

In general, wound healing involves a series of cellular and molecular events which initiates after injury of the epithelial lining and disruption of the underlying vasculature with an increased influx of platelets and inflammatory cells, in the primary stages predominantly neutrophils, followed by macrophages and T-lymphocytes (41). These platelets and inflammatory cells are capable of releasing many growth factors and cytokines, and molecules like fibrin and fibronectin to close and hold together the wounded tissue (41). Currently, neutrophils are believed to act as first-line of defense against invading micro-organisms and the elimination of other foreign material by the release of anti-microbiologic peptides like defencins, reactive oxygen species (ROS) and proteinases, but they are also responsible for so called "friendly–fire" leading to damage on viable surrounding tissues (29). The next cell to appear is the macrophage, which is the key-orchestrator of tissue repair processes (41, 42). Cytokines and growth factors released by surrounding epithelial cells, macrophages and T-lymphocytes attract (myo-) fibroblast to the wounded area which

start to release additional growth factors and cytokines, especially TGF-β_1, responsible for the synthesis and consecutive deposition of extracellular matrix (ECM) products such as collagen subtypes I, III V, VIII, and proteoglycans (37, 40, 43). Neo-vascularization and angiogenesis is initiated by the release of angiogenic growth factors like vascular endothelial growth factor (VEGF) by macrophages and other immune cells in response to a hypoxic environment. VEGF stimulates endothelial cell proliferation, migration and new tube formation (44). Taken together this environment of a rich cocktail of growth stimulatory cytokines and growth factors, (myo)-fibroblast derived new extracellular matrix networks and adequate capillaries facilitate proliferating epithelial cells to migrate which leads to closure of the wound (41). Controlling inflammation and (myo-) fibroblast growth is as important as initiating above events, thus minimizing additional damage and abnormal wound healing with scarring and excessive fibrogenesis (41).

Deregulated repair processes

Ongoing chronic inflammation with repetitive cycles of tissue damage and repair can lead to severe scarring abnormalities, predominantly by excessive deposition of ECM products by myo-fibroblasts. Within the airways, the bronchial epithelium, sub-epithelial myo-fibroblasts, airway smooth muscle cells are major cell types involved in tissue repair processes and excessive stimulation can lead to airway wall remodeling with subepithelial fibrosis (8). Although it is becoming clear that many cytokines and growth factors are involved. Among these are the pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukine-8 (IL-8) and IL-1β. These cytokines play an important role in chemotaxis of neutrophils and macrophages to the airway wall and lumen, and are also involved in bronchial epithelial survival and repair (36, 43, 45-47). TNF-α is a multi-functional cytokine, which can be induced in epithelial cells and inflammatory cells by cigarette smoke (48). It can induce neutrophil degranulation, release of proteolytic enzymes and mucus cell metaplasia with mucus hypersecretion (48). Furthermore, TNF-α has the ability to induce many additional products among them TNF-α itself, IL-8, IL-1β. Indeed, increased levels of TNF-α and IL-8 are found in sputum, bronchial alveolar lavage (BAL) fluid, bronchial epithelium and airway of COPD subjects as compared to non-symptomatic smokers (48).

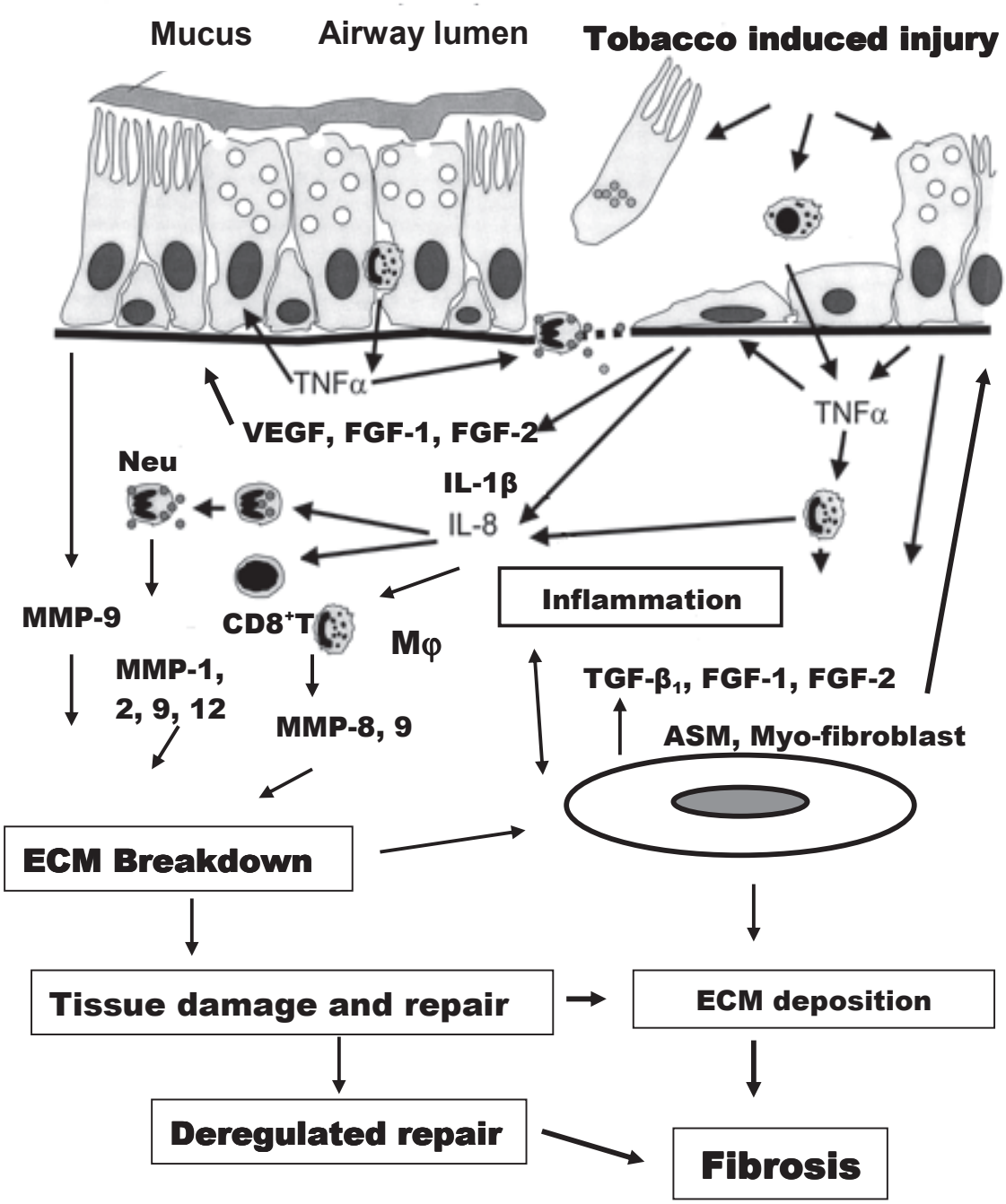

Figure 1.5 **A scheme of cytokine and growth factor actions in human airways.** On triggering, eg, with tobacco smoke, epithelial cells are damaged, epithelial cells and resident macrophages produce inflammatory mediators such as tumor necrosis factor (TNF)-α interleukin (IL)-1β, IL-8. In turn, inflammatory mediators-stimulate migration of monocytes/macrophages, neutrophils, CD8 positive T-lymphocytes to the airway. Both TNF-α and IL-8 can cause degranulation of neutrophils with production and release of serine-proteinases, metalloproteinases (MMPs) as well as free radicals that can cause matrix and epithelial damage. In turn, TNF-α and released growth factors like vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF-1 and FGF-2) orchestrate epithelial repair. Ongoing inflammation and tissue breakdown trigger the release of growth factors like transforming growth factor-β_1 (TGF-β_1) inducing ECM production by myo-fibroblasts. Repetitive tissue damage and repair can lead to excessive ECM deposition and subepithelial fibrosis. Neu = neutrophil; Mφ = macrophage $CD8^{+ve}$ T = CD8 positive T-lymphocytes. Based on Refs. (1, 48).

Furthermore, a variety of growth factors including platelet–derived growth factor-BB (PDGF-BB) and vascular endothelial growth factor (VEGF), transforming growth factor-β_1 (TGF-β_1) and fibroblast growth factors (FGFs) that are released from the epithelium, neutrophils, macrophages and myo-fibroblast may contribute to the pathogenesis of COPD (49-52). Although many other cytokines and growth factors can contribute, some of the important cellular and molecular events in the epithelial repair process and possible mechanism leading to sub-epithelial fibrosis in COPD are summarized in Figure 1.5. The major sources, target cells and effects for several growth factors implicated in chronic lungs diseases are listed in Table 1.2. Taken together, growth factors could therefore be important players in airway remodeling in the development of COPD.

1.5 The Role of cytokines and growth factors in COPD

Fibroblast growth factors

The fibroblast growth factor family is implicated in a wide variety of patho-physiological conditions including systemic hypertension, ischemic heart disease and interstitial lung fibrosis and may as well be involved in chronic inflammation, fibrosis and tissue repair during airway remodeling in COPD (53-56). The fibroblast growth factor family currently consists of at least than 23 members of which FGF-1 (acidic FGF) and FGF-2 (basic FGF) were the first discovered and are the most important ones, which share approximately 53 % sequence homology (57). FGFs play a role in morphogenesis, angiogenesis, tissue and ECM remodeling during normal development and disease states in almost every organ (57-61). In the lungs, FGF-1 and FGF-2 are produced by many cell type including airway epithelium, alveolar macrophages and mast cells, (myo-)fibroblast, airway smooth muscle cells (38, 62-64). Next to FGF-1 and FGF-2, two important members FGF-7 (keratinocyte growth factor) and FGF-10 are predominantly involved in development and maturation of the lungs (60).

Table 1.2

Major growth factors in airway remodeling			
Growth factor	Source	Target	Function
FGF-1	ECM Fibroblast	Fibroblast ASM VSM Epithelium	Proliferation, Collagen production, Proliferation Collagen production
FGF-2	ECM Endothelial cell ASM VSM Macrophages	Endothelial cell Fibroblast ASM VSM Epithelium	Proliferation Proliferation Proliferation Proliferation Proliferation
VEGF	Epithelium ASM Endothelial cells VSM Macrophages ECM	Endothelial cell Epithelial cells Fibroblast Macrophages	Proliferation, Migration Proliferation Proliferation, Recruitment Recruitment
TGF-ß	ECM Platelets, Macrophages Fibroblast ASM	Fibroblast ASM VSM Endothelial cell Epithelium Neutrophil, T-lymphocytes Monocyt/macrophage	ECM production, Recruitment ECM production ECM production Differentiation, ECM production Apoptosis Differentiation, ECM production Chemotaxis
PDGF	Platelets, Endothelial cell Macrophages Fibroblast ASM Epithelium	ASM Epithelium Fibroblast	Proliferation Proliferation Recruitment, Proliferation
IGF-1	ECM Fibroblast	Fibroblast ASM	Proliferation and Differentiation Collagen synthesis
IGF-2	ECM Fibroblast	Fibroblast	Proliferation, Differentiation Collagen synthesis

Abbreviations; Transforming growth factor beta (TGF-ß), Fibroblast growth factor (FGF), Vascular endothelial growth factor (VEGF), Platelet-derived growth factor (PDGF), Insulin-like growth factors (IGF), Airway and Vascular smooth muscle (ASM and VSM), Extracellular matrix (ECM). References (37, 38, 64-66).

Their cellular responses are very divers ranging from proliferation, migration, differentiation, cell viability as well as either stimulation or inhibition of ECM production. Target cells of FGF-1 and FGF-2 include epithelial cells, fibroblasts on which they act as potent mitogen as well as inducers of ECM synthesis (38, 67). Although both FGFs have mitogenic effects on epithelial cells, fibroblasts and on cells of smooth muscle origin, FGF-1 has been associated with higher proliferation of epithelial cell lineage, while FGF-2 is generally more potent than FGF-1 on cells of mesenchymal origin like fibroblast and smooth muscle cells. Basic FGF induces vascular smooth muscle cells and endothelial cell proliferation, and is therefore also considered as a potent factor in angiogenesis (68, 69).

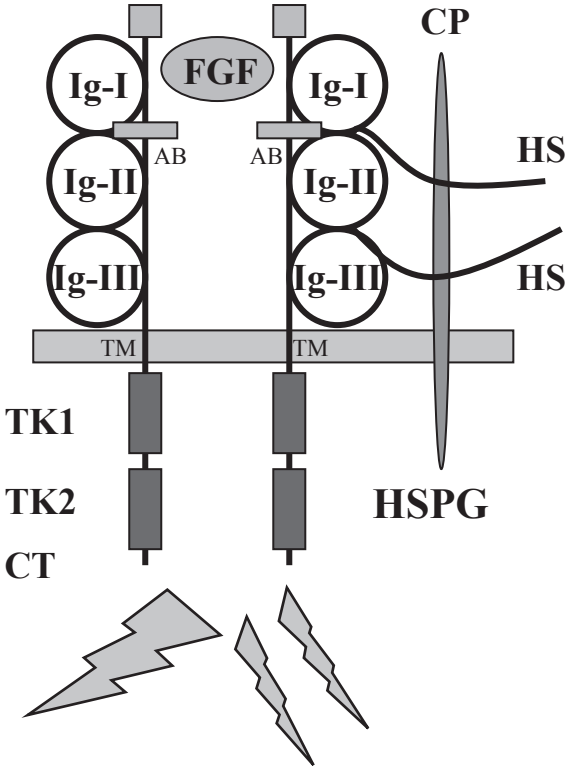

Figure 1.6 **FGF receptor signaling.** Multiple levels of regulation of FGF-mediated cellular responses exist. A; Selection of the ligand: 23 different FGF-ligands can bind to the FGF receptors. Predominantly FGF-1, FGF-2, FGF-7 and FGF-10 are expressed in the embryonic, postnatal and normal or pathological adult lungs. B; Selection of the FGF receptor and co-receptors: there are four FGF tyrosine kinase receptors FGFR-1 to FGFR-4 and heparan sulphate proteoglycans (HSPGs) co-receptors such as membrane-associated Syndecans (1-4), Glypicans (1-6) and Perlecan. The expression of different receptors and co-receptors can influence the cellular responses to the FGFs. C; Selection of multiple signaling pathways: many intracellular signaling pathways have been described. Abbreviations; TK = tyrosine kinase domain; Ig = Immunoglobulin-like domain; AB = acid box, ligand binding site; TM = trans membrane domain; HS = heparan sulphate chains; CP = HSPG core protein. Based on Refs. (58, 61).

Fibroblast growth factors exert their biological effects via binding to four high-affinity, transmembrane tyrosine-kinase receptors designated FGFR-1 through FGFR-4 (58). Distinct FGF subtypes bind with different affinity to the various FGF receptors. Alternative splicing and regulated protein trafficking further modulate the intra-cellular events and resultant response initiated by FGF ligand-receptor interaction (58). Additional regulatory binding sites for FGFs consist of heparan-sulphate proteoglycans (HSPGs) that appear to be macromolecular receptors which can modulate the effects of FGFs, both stimulatory and inhibitory depending on the heparan-sulphate side chain as well as the proteoglycan core protein (70). HSPGs are part of the ECM and are located on the surface of most cell membranes closely linked with the high affinity tyrosine-kinase receptors (70). Figure 1.6 schematically summarizes the interactions of FGFs with their tyrosine–kinase receptors and HSPGs. Unlike the other members of the FGF family, the acidic and basic FGF lack cytoplasmic sequences for extracellar export. In this regard, the growth factors could be released during cell lysis and the HSPGs could act as a reservoir of growth factor that can be released in a enzymatic regulated manner during ECM breakdown (71).

Additionally, fibroblast growth factor family members are implicated in pathological conditions with tissue remodeling and lung fibrosis (55, 62, 72). Barrios and coworkers (55) showed FGF-1 and FGFR-1 expression in experimentally induced pulmonary fibrosis. Becerril and colleagues found that FGF-1 overexpression in the lung fibroblasts results in down-regulation of collagen synthesis and up-regulation of collagenases, which may protect against fibrosis (72). In a recent study production of FGF-2 from mast cells and the expression of FGFR-1 (Flg) and FGFR-2 (Bek) protein were positively linked to idiopathic pulmonary fibrosis (62). FGF-2 and also PDGF have been implicated in the pathogenesis of obliterative bronchiolitis after transplantation (73).

In the normal pulmonary vasculature, FGF-1, FGF-2 and FGFR-1 are constitutively expressed in the media (vascular smooth muscle cells) of pulmonary vessels and FGF-2 is also found in endothelial cells (63). Singh and colleagues demonstrated that increased expression of FGF-2 in vascular smooth muscle and endothelium precedes arterial enlargement in response to increased arterial blood flow *in vivo* (54). Furthermore, Bryant et al recently found that administration of FGF-2 could be protective against a decrease in vessel luminal area and wall thickening in response to altered blood flow and that this inhibitory effect could be blocked by

anti-FGF-2 neutralizing antibodies (74). Taken together, FGFs could therefore be important players in airway and vascular remodeling in the development of COPD.

Vascular endothelial growth factor

A variety of angiogenic growth factors such as vascular endothelial growth factor (VEGF) and FGF-2 released from various cell types of airway as well as vascular walls have the potential to contribute to the pathogenesis of COPD. One of the potent proteins involved in vascular remodeling is vascular endothelial growth factor (VEGF). The VEGF family currently comprises six members (VEGF-A to F), of which the originally identified VEGF-A_{165} variant is the predominant form of five additional spliced variants (75). Like FGFs, VEGFs are heparin-binding proteins and acting via their high affinity, transmembrane receptors VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1), (75). The receptors belong to the family of tyrosine kinases and are predominantly expressed by endothelial, VSM cells and epithelial cells (75). Recent studies indicate that VEGF is expressed in the lung by bronchiolar, submucosal glandular and alveolar type I and II epithelial cells, alveolar macrophages, airway and vascular smooth muscle (ASM and VSM) cells as well as myo-fibroblast in fibrotic lung lesions (76-78).

VEGF promotes an array of responses in the endothelium including hyperpermeability, endothelial cell proliferation and angiogenesis with new vessel tube formation in vivo (75, 79). Moreover, the expression of VEGF can be induced under a variety of pathophysiological conditions, including pulmonary hypoxia and pulmonary hypertension with increased sheer stress (76, 79). Hypoxia and pulmonary hypertension are pathological features often seen in advanced COPD patients and increased VEGF expression under influence of hypoxia-inducible transcription factors (HIFs) may contribute to increased and abnormal proliferation of endothelial and VSM cells in pulmonary vessels leading to vascular remodeling (8).

The role of VEGF and its receptors in the lungs of COPD patients remains unclear. Vascular endothelial growth factor (VEGF) and its receptor 2 (VEGFR-2) are involved in proper maintenance, differentiation, and function of endothelial as well as epithelial cells. Voelkel and co-workers demonstrated that VEGFR-2 blockade in combination with chronic hypobaric hypoxia destroyed lung capillaries by inducing endothelial cell apoptosis and at the same time caused precapillary pulmonary arteries occlusion by proliferated endothelial cells (79-82). Furthermore, they observed that

emphysematous patients have decreased levels of VEGF messenger RNA and protein as well as decreased expression of VEGF receptor 2, KDR/flk-1(83). In this recent study, decreased VEGF and KDR/flk-1 expression was associated with endothelial and also epithelial cells death in alveolar septa due to a decrease of endothelial cell maintenance factors which may be part of the pathogenesis of emphysema (83). Thus, the expression of VEGF may be protective against signals leading to apoptosis such as toxic agents from tobacco smoke.

Alternatively, abundance of VEGF and receptor mRNAs (Flt-1 and KDR/Flk) decreased in endothelial cells during hyperoxia, possibly secondary to the loss of endothelial cells by apoptosis. This also indicated that VEGF functions as a survival factor in the normal adult rat lung, and its loss during hyperoxia contributes to the pathophysiology of oxygen-induced lung damage (84).

Although the role of VEGF in the vascular biology is thoroughly studied, it has become clear that VEGF and receptors are involved in various other cellular events as well, including epithelial proliferation and survival, and the recruitment of mast cells, neutrophils and macrophages to sites of fibrosis (79, 81, 85). Taken together, VEGF and its receptors could therefore also be important players in airway and vascular remodeling in the development of COPD.

Transforming growth factors

TGF-β and receptor expression in lungs have been associated with asthma, chronic bronchitis, idiopathic pulmonary fibrosis (86, 87). In patients with chronic bronchitis or COPD TGF-β_1 mRNA and protein are observed in bronchial and bronchiolar epithelium, macrophages, mast cells and pulmonary vessels and increased TGF-β_1 protein levels are found in the epithelium of COPD patients as compared to smoking controls (50, 88).

TGF-β is a multifunctional polypeptide growth factor, which is involved in inflammation and connective tissue synthesis. TGF-β belongs to a large superfamily currently including more than 30 members, which also include bone morphogenic proteins, inhibins and activins (37). Three different mammalian isoforms exist (TGF-β_1 to -β_3) of which the TGF-β_1 isoform is the most potent and binds to at least three high affinity receptors (TGFβR I-III), (37). TGF-β is released as a biologically inactive precursor consisting of a dimer with the N-terminal pro-region, latency-

associated peptide (LAP), to which inactive TGF-β_1 is bound. Furthermore, latent TGF-β_1 complex can bind to latent binding protein-1 (LTBP-1) that in turn binds to the extracellular matrix which serves at a reservoir for active TGF-β_1 (89). In addition, the release of latent TGF-β_1 from the extracellular matrix is a consequence of cleavage of LTBP. Activation and release of TGF-β_1 dimer is achieved in vivo by enzymatic cleavage from intracellular and extracellular latent TGF-β_1 stores by serine proteases as well as various metalloproteinases (37, 90).

Its actions highly depend on the target cell-type or situation present. The TGF-β superfamily is important in cell development and differentiation and proliferative regeneration (37). In epithelial and endothelial cells TGF-β_1 is usually associated with terminal differentiation, growth inhibition and even apoptosis. During wound healing TGF-β_1 is involved in regeneration (37). In (myo-) fibroblasts, smooth muscle cells and other cells of mesenchymal origin stimulation of proliferation, synthesis of ECM proteins including collagens, elastin, proteoglycans and fibronectin are induced by TGF-β_1 (37, 91, 92).

1.6 Extracellular matrix biology in COPD

Extracellular matrix proteins in the lungs

The extracellular spaces within tissues and cells are filled with organized extracellular matrix (ECM) proteins that are important for structural integrity, strength as well as elasticity of tissues. The major components of the ECM consists of fibrous proteins like collagens, elastin and fibrillin, proteoglycans such as syndecans, glypecan, perlecan and decorin as well as adhesion molecules like fibronectin and laminins. It has been become clear that the ECM molecules play important roles in cell signaling and cellular activities. Fibronectin and laminin well as some collagens are bound to cells through specific binding sites or receptors, the integrins, of which more than 20 different subtypes are identified. These integrin receptors are heterodimeric transmembrane receptors, consisting one α and β chain, which specially bind different ECM molecules (65, 93). Furthermore, fibronectin, on the other hand, has specialized domains for different collagens, so that the various components of the ECM are tightly interconnected with each other and with cells. Currently, more than 20 collagen subtypes are identified, of which the subtypes I and III are the most abundant forms, found throughout the interstitial spaces and in between cells of many tissues

(65, 93, 94). Within the lungs, these collagen subtypes are deposited in the interstitium of airway wall, beneath the epithelial lining, and within the blood vessels and alveolar septa (95, 96). Collagen IV and laminin are the main constituents of cellular basement membranes, connecting epithelial or endothelial cells, functioning as outward cellular linings, with collagen subtypes I, III and VI inserting within the underlying interstitium (97, 98).

Mature processed collagen molecules aggregate to form larger triple-stranded helical fibrous structures and help to form the ECM with other components (65, 93). Therefore, normal structural type I and III collagen production and deposition in the ECM to make normal physiological connective tissue is highly regulated by cytokines and growth factors like TGF- β, TNF-α and FGF-2 and their transcriptional as well as post-translational modulatory steps (65, 93). Abnormalities in any of regulatory step may cause defective and accumulation of collagen in ECM, which in turn causes pulmonary fibrosis (94).

ECM production and fibrosis

Stimulation of ECM production by TGF-β_1 appears to be normal for either mesenchymal or epithelial cell origin. In fibroblasts and smooth muscle cells TGF-β_1 also promotes expression of actin, myosin, smooth muscle actin and cell adhesion molecules such as integrins, including one specific and important combination $\alpha_2\beta_1$ integrin receptor, also known as the collagen receptor. Moreover, TGF-β_1 down-regulates the expression of matrix degrading enzymes (matrix metalloproteinases), specifically MMP-1, MMP-3 and induces the expression of protease inhibitors, such as tissue inhibitor of matrix metalloproteinase (TIMPs), (37). Taken together, the observations above inextricably link the processes of ECM guided degradation and migration, ECM production and scarring contraction of myo-fibroblasts to the functions of TGF-β_1. As indicated by early reports and many follow-up studies the most important concepts in the onset and continuation of fibrosis is the presence of TGF-β_1 at areas with injury to the epithelium and underlying basements membranes (86, 87).

ECM breakdown

Historically, the role of compounds from neutrophils and also macrophages have been implicated in the pathogenesis of COPD, based on the relation between α_1-antitrypsin deficiency and the predisposition for the development of emphysema in a rare number of patients (42, 99, 100). α_1-antitrypsin or secretory leukocyte proteinase inhibitor (SLPI), inhibits neutrophil serine-proteinases, especially elastase, which can cleave elastin, thus causing damage to alveoli and eventually emphysema (29, 30). Neutrophils store high amounts of serine proteinases and in addition at least two MMPs, MMP-8 (neutrophil collagenase) and MMP-9 (gelatinase B), Zn^{2+}-ion catalyzed enzymes of which currently more than 20 members have been identified (99, 101). These MMPs can cause degradation of most components of the extracellular matrix upon neutrophil activation (100). Furthermore, MMPs can be secreted by macrophages. The release and action of MMPs are strictly regulated by for instance growth factors and cytokines and especially by enzymes called tissue inhibitors of metalloproteinases (TIMPs), of which currently four members have been identified (102). Furthermore, MMPs can mediate the release and activation of ECM-bound (e.g. TGF-β1, FGFs, EGF, IGF-1 and TNF-α) or cell membrane-bound (IL-6 and TNF-α) growth factors and cytokines, thereby promoting ongoing inflammation and tissue remodeling (100). However the opposite, degradative inactivation of IL-1β has also been described (100). All of these actions can contribute to pathologic tissue remodeling including inflammation and cellular proliferation as well as ECM breakdown and deposition during COPD. Indeed, subjects of emphysema showed increased levels of MMP-1 (interstitial collagenase 1), MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in macrophages, alveolar type II cells and fibroblasts as compared to non-emphysematous controls (99). A similar approach, using bronchial tissue from COPD subjects, immunolocalized several MMPs, including MMP-1, MMP-2, MMP-9 and also MMP-8 and MMP-13 (interstitial collagenases 2 and 3), and found increased expression of MMP-1 and MMP-2 levels in bronchial epithelial cells, luminal and interstitial macrophages (103). Furthermore, MMP-9 and its inhibitor TIMP-1 were upregulated in sputum of chronic bronchitis patients (104). Taken together, these MMPs can degrade collagen and elastin, the major components the extracellular matrix, thereby implicating macrophages and neutrophils as important contributors to excessive tissue breakdown and injury in COPD and predisposing regenerative tissue towards deregulated repair with fibrosis.

1.7 Angiogenesis and vascular remodeling in COPD

COPD patients with moderate to severe disease display elevated pulmonary vascular pressures during exercise and pathological changes in the pulmonary circulation (8, 105). Wright et al. (105, 106) demonstrated increased wall thickness of small (< 500 μm) pulmonary vessels in COPD subjects as compared to non-symptomatic smokers, which was correlated with the severity of the disease (as indicated by a decline in FEV_1). Additionally, COPD patients with mild to moderate COPD showed intimal thickening and severe subjects of the disease also developed medial thickening.

In COPD, alveolar hypoxia can cause pulmonary vasoconstriction and, if the hypoxic stimulus persists, pulmonary vascular remodeling, of which increased muscularization of small arterial branches is the most striking feature (18). With sustained vasoconstriction of pulmonary arteries, arterioles and veins, the medial vascular smooth muscle (VSM) extends distally to vessels normally devoid of smooth muscle (18). Intimal thickening and emergence of smooth muscle cells within the intima of small pulmonary arterial branches has been attributed to a chronic inflammatory process accompanied with fibrosis in part similar to arteriosclerosis in cardiovascular disease (107, 108).

Recently, Peinado et al. showed also intimal but not medial thickening in the vasculature of mild COPD patients compared to non-smoking controls (109). Furthermore, observations from the same group indicated that muscular pulmonary and bronchiolar arteries have increased adventitial infiltration of inflammatory cells, predominantly $CD8^{+ve}$ T-lymphocytes and displayed VSM heterogeneity in relation to desmin as well as intimal thickening that was correlated to the amount of total collagen deposition (110, 111). The infiltration of the vascular wall with inflammatory cells may contribute to vascular wall thickening. Finally, loss of the pulmonary vascular bed by emphysema has been suggested to lead to the formation of new vessels (18). Thus, several phenomena acting in concert in COPD result in pulmonary vascular remodeling. Yet, little is known about the molecular mechanisms underlying these processes in the context of COPD.

Angiogenesis

Mature endothelial cells are quiescence cells with an extremely low proliferative index. Smoke induced injury with hypoxia, however, induce VEGF-A mRNA expression via hypoxia inducible transcription factors (HIF 1 to 3), (75, 112). This initiates angiogenesis by increasing endothelial permeability and stimulates endothelial cells to secrete several proteinases, such as MMPs including collagens and elastin degrading MMP-1, MMP-2, MMP-3 and MMP-9, and heparinase acting on proteoglycans (44). This, in turn, leads to ECM breakdown and the liberation of additional growth factors, predominantly VEGF-A itself as well as FGF-2 and insulin-like growth factor-1 (IGF-1) sequestered in within the surrounding matrix (44, 75). Proliferating endothelial cells migrate to distant sites in wounded or inflamed tissue, which is predominantly guided by actions of VEGF and FGF-2 in close contact with the collagen and heparan-sulphate proteoglycan matrix, thus resulting in new tube formation (70).

Vascular remodeling

Of great importance is the recruitment of a stable vascular smooth muscle coating to newly formed vessels. This is initiated by VEGF in combination with angiopoietins produced by endothelial cells, of which currently four ligands are known (ANG-1 to ANG-4) that bind to two receptors expressed by endothelial cells, tie-1 and tie-2 (113). Binding ANG-1 to tie-2 receptor induces endothelial cells to recruit fibroblasts or VSM, whereas ANG-2 binding to tie-2 repels this event (113). TGF-β_1 and TGF-βR2 are involved in vessel maturation by inhibiting endothelial cell proliferation and inducing smooth muscle differentiation and stimulating of ECM deposition by VSM cells and fibroblast, thereby solidifying the vessel wall (44).

Pathological arteriogenesis involves hypoxia, tissue ischemia, increased sheer stress, which can inflicts damage to endothelial and VSM cells (44). Inflammatory cells such as monocytes, macrophages and $CD8^{+ve}$ T-lymphocytes infiltrate the vessel wall constitutively. Inflammation can cause additional damage to the vessel wall. Endothelial and VSM cells release growth factors such as FGF-2, PDGF and TGF-β_1 in response to inflammatory mediators. Eventually deregulated repair can lead to fibrotic tissue deposition and vascular remodeling (44). Taken together, vascular remodeling and angiogenesis in peripheral, as well as in central airways could also be associated with the pathogenesis of COPD.

1.8 ASM cells in airway remodeling

Concepts of the contribution of human airway smooth muscle (ASM) cells to pathophysiological events during chronic airway diseases like asthma and COPD have drastically changed. Historically, ASM cells were considered as structural cells implicated in the regulation of producing immediate airway narrowing or widening merely by contraction and relaxation. However, the ASM cell can participate in inflammatory responses, release many chemotactic cytokines and growth factors, present necessary receptors and adhesion molecules and produce ECM components as well as ECM degrading proteases (114-116).

Heterogeneity and phenotypic plasticity in ASM cells

Recent studies indicate that ASM cells are apparently functionally and structurally divers and that heterogeneity and plasticity in phenotypes exists, which equip ASM cells with the potential to regulate airway lumen diameter both transiently, via reversible contraction, as well as chronically via remodeling by muscle hypertrophy (117). Phenotypic plasticity was first described in differentiated, cultured vascular smooth muscle cells derived from the medial layer of large elastic arteries (118). Mature vascular and also airway smooth muscle cells acquire an "immature" synthetic phenotype when incubated in serum-enriched culture, exhibiting a high proliferative index and loss of contractile elements and their associated proteins, defined as modulation (118, 119).

For example, pro-inflammatory mediators such as TNF-α and IL-1β were potent inducers of interleukin (IL)-8 release by ASM cells and together they synergistically augmented IL-8 release. IL-8 is a C-X-C chemokine that potently chemoattracts and activates neutrophils. Therefore, in addition to its contractile responses, airway smooth muscle cells have synthetic and secretory potential with the release of IL-8 and subsequent recruitment and activation of neutrophils in the airways.

The reversion of primary cultured smooth muscle cells to a contractile state also occurs after cultures grow to confluence or undergo serum starvation (maturation), which is marked by an increase in myofilaments and contractile apparatus-associated protein content (120). Recently, intermediate subtypes have

been identified. The results from a recent study showed that IL-4 and IL-13 increased alpha-smooth muscle actin expression in myo-fibroblasts and thus that IL-4 and IL-13 are capable of inducing the phenotypic modulation of human lung fibroblast to myo-fibroblasts (121). This can influence the interaction of myo-fibroblast with the surrounding collagen matrix, modulating there contractile properties as indicated by a study investigating the contraction of these cells embedded in collagen I type gel matrix in response the cytokines (122).

Additionally, a distinct subset of ASM cells has been identified with a fully contractile phenotype, elongated morphology, abundant contractile apparatus proteins such as smooth muscle α and γ-actin, smooth muscle myosin heavy chain, SM22 and α_1-integrin, reacquisition of pharmacological responsiveness to acetylcholine (116, 120). Additionally, contractile myocytes show a time-dependent subcellular reorganization of the contractile apparatus in response to changes in muscle length defined as mechanical plasticity (117). Figure 1.7 summarizes the important features of ASM cell heterogeneity, phenotypic and mechanic plasticity.

Proliferation of ASM cells

Since culture of human ASM cells was possible and since the discovery of the synthetic, highly proliferative ASM cells, the effect of mitogens and the signal transduction pathways leading to proliferation have studied intensively (114). The effects of mitogens for ASMC are mediated through at least two distinct receptor systems: Receptor tyrosine-kinase (e.g. platelet derived growth factor, epidermal growth factor as well as acidic and basic FGF) and G protein-coupled receptors (e.g. thrombin), (114, 123). Also the effects of TGF-β on ASM cell proliferation and ECM production have been thoroughly studied.

Black and colleagues found that 24 hours of incubation with TGF-β_1 decreased DNA synthesis, whereas 48 and also 72 hours increased DNA synthesis and proliferation in cultured bovine ASM cells (125). Interestingly TGF-β_1 inhibited 10% FBS induced DNA synthesis in sparsely seeded bovine ASM cells, whereas DNA synthesis was increased after 48 hours of TGF-β_1 treatment in the presence of only BSA in confluent grown cells (126). Taken together, these studies demonstrated that TGF-β and TGF-β receptors are present on ASM cells and that TGF-β_1 modulates the effects on proliferation with a condition-dependent nature (125-129).

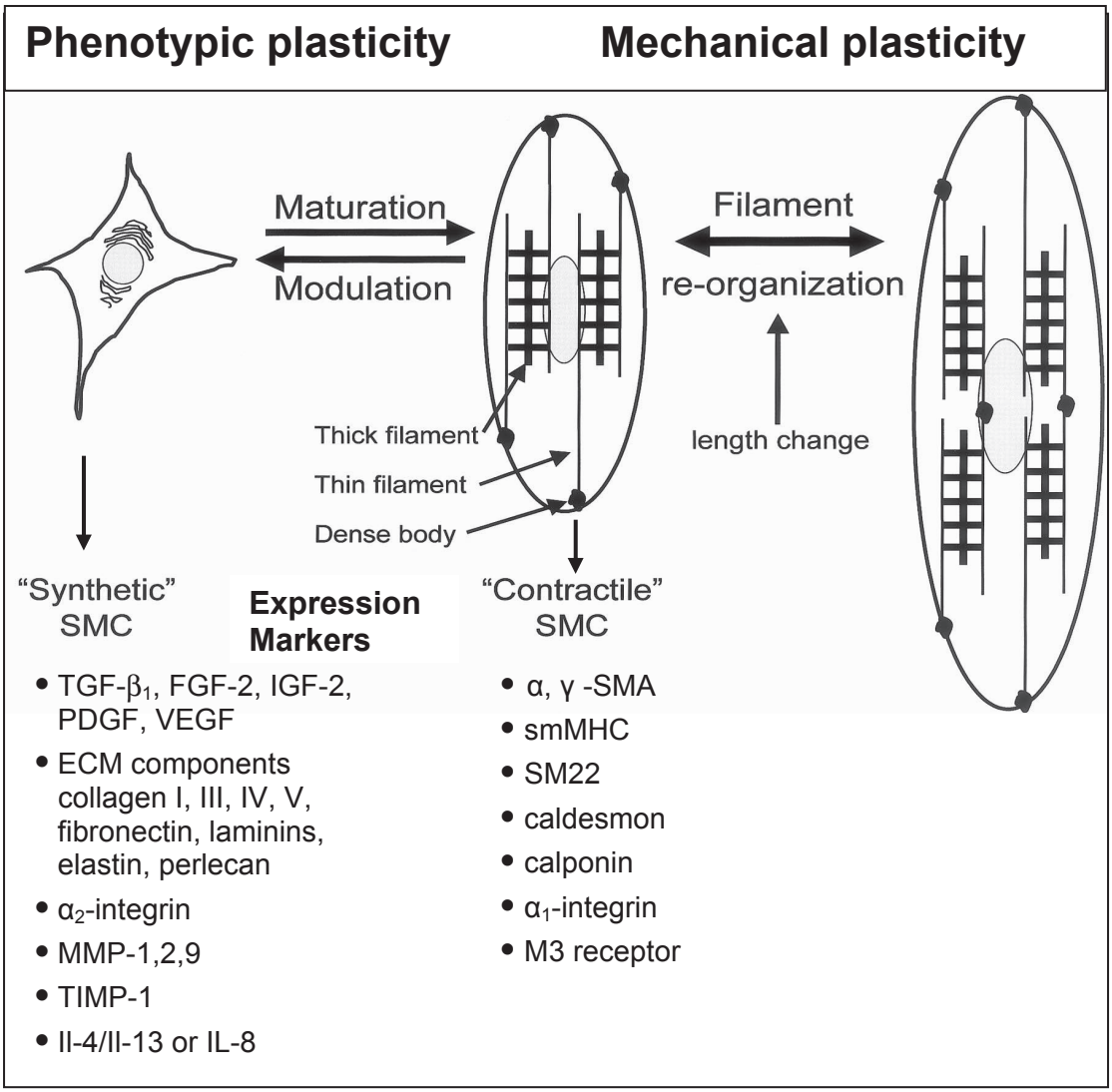

Figure 1.7 **Schematic representations showing the association of phenotypic and mechanical plasticity on airway smooth muscle.** Phenotypic plasticity results from reversible modulation and maturation of airway smooth muscle cells (ASMC) between a synthetic and contractile state associated with differential gene expression. Mature ASM cells acquire an "immature" synthetic phenotype when incubated in serum-enriched culture, exhibiting a high proliferative index and loss of contractile elements such as smooth muscle α and γ-actin (α, γ-SMA), smooth muscle myosin heavy chain (smMHC), SM22 and α_1-integrin, and loss of pharmacological responsiveness to acetylcholine via muscarinic M3) receptor. ASM cells can produce growth factors, extracellular matrix products as well as matrix degrading enzymes (matrix metalloproteinases, MMPs) and their inhibitors (TIMPs). Integrins are involved in the interaction of ASM cells with the binding to collagen I in the ECM. The binding of $\alpha_1\beta_1$ to collagen I results in an almost complete arrest of collagen synthesis, whereas the binding to $\alpha_2\beta_1$ integrin leads to induction of growth factors like TGF-β_1, MMP-1 as well as collagen gene expression (synthetic phenotype). Mechanical plasticity occurs in contractile myocytes as the result of time-dependent subcellular reorganization of the contractile apparatus in response to changes in muscle length. Adapted from Refs. (114, 117, 124).

ASM and extracellular matrix

Khalil et al. demonstrated that the release of biological active TGF-β_1 under influence of plasmin can induce ASM cells to synthesize pro-collagen I in an autocrine manner (130, 131). Furthermore, human ASM cells produce many other ECM components including collagen types I, III, IV and V, fibronectin, laminins, elastin and HSPGs (e.g. perlecan and syndecan) (132). In addition, ASM cells can secrete MMP-1 (interstitial collagenase 1), MMP-2 (gelatinase A) and MMP-9 (gelatinase B) as well as TIMP-1 (124, 132). Hirst and colleagues showed that cell-matrix interactions, in addition to growth factors, could have important effects on ASM cell proliferation and phenotype. In this study the authors showed that ASM cells cultured on collagen I or fibronectin matrix have increased proliferation whereas ASM cells grown on laminin proliferate more slowly yet express contractile proteins.

Evidence for airway SMC heterogeneity and plasticity *in vivo* is indicated by observations in asthma and also COPD of accumulation of synthetic myocytes (myo-fibroblasts) in the submucosal region of the bronchial wall as well as significant increased airway smooth muscle mass possibly through hypertrophy and hyperplasia (28, 133, 134). Taken together, the ASM cell can be considered as an important cell type in the progression of airway remodeling in COPD.

1.9 Aims of the thesis

Chronic obstructive pulmonary disease is characterized by airflow limitation that is irreversible and without smoking cessation usually progressive. The disease is associated with an abnormal inflammatory response of the lungs to noxious particles and gases. Exposure to particles from the tobacco smoke inflicts damage onto a variety of structures at several levels in the lungs from conducting larger airways, respiratory airways to alveolar regions as well as in the pulmonary and bronchiolar vasculature. Although subtle local differences may exist in the lungs the common feature of pathological processes in COPD is; chronic challenge lead to repetitive cycles of tissue injury with inflammation and repair, which may result in tissue remodeling and structural abnormalities that, in turn, can cause airflow limitation. Although definite progress has been made in the descriptions of pathological alterations in COPD at the histology level, the underlying molecular events remain largely unknown. We, therefore, hypothesized that the observed structural alterations

may arise from alterations in gene expression in the affected cell populations. We investigated the role of growth factors and extracellular matrix in the development of airway and vascular wall structural changes in COPD at the molecular level.

The specific aims of the studies described in this thesis are:

1. To investigate the structural alterations and the role of the fibroblast growth factor/receptor (FGF/FGFR) system in the pulmonary vasculature between non-COPD and COPD patients in COPD (chapter 2).
2. To investigate the role of vascular endothelial growth factor (VEGF) and its receptors Flt-1 and KDR/Flk-1 in airway and vascular remodeling during COPD (Chapter 3).
3. To elucidate the role of the FGF/FGFR system during structural remodeling of central airways in patients with COPD (Chapter 4).
4. To describe the distribution of various extracellular matrix components including collagens subtypes I, III, IV, fibronectin and laminin in the central airways of non-symptomatic smokers and COPD patients in relation to airway and vascular remodeling (Chapter 5).
5. To investigate the proliferative response and ECM synthesis by airway smooth muscle cell in reaction to growth factors TGF-β_1, FGF-1 and FGF-2 *in vitro*: as a contribution to accumulation of airway smooth muscle mass by hypertrophy and/or hyperplasia during COPD *in vivo* (Chapter 6).

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Chapter 2

Fibroblast growth factors and vascular remodeling in COPD

Adapted from:

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2.1 Summary

Important characteristics of Chronic Obstructive Pulmonary Disease (COPD) include airway and vascular remodeling, of which the molecular mechanisms are poorly understood. We assessed the role of fibroblast growth factors (FGF) in pulmonary vascular remodeling by examining the expression pattern of FGF-1, FGF-2 and their receptor, FGFR-1 in peripheral area of the lung tissues from patients with COPD (FEV_1 ≤75%; n=15) and without COPD (FEV_1 ≥85%; n=13). Immunohistochemical staining results were evaluated by digital video-image analysis as well as by manual scoring. FGF-1 and FGFR-1 were detected in vascular smooth muscle (VSM), airway smooth muscle (ASM) and airway epithelial cells. FGF-2 was localized in the cytoplasm of airway epithelium and in the nuclei of ASM, VSM and endothelial cells. In COPD cases, an unequivocal increase in FGF-2 expression was observed in VSM (3 fold, p=0.001) and endothelium (2 fold, p=0.007) of small pulmonary vessels with a luminal diameter under 200 μm. In addition, FGFR-1 levels were elevated in the intima (1.5 fold, p=0.05). VSM cells of large (>200 μm) pulmonary vessels showed increased staining for FGF-1 (1.6 fold, p<0.03) and FGFR-1 (1.4 fold, p<0.04) in COPD. Pulmonary vascular remodeling, assessed as the ratio of α-smooth muscle actin staining and vascular wall area with the lumen diameter, was increased in large vessels of COPD (p=0.007) and was inversely correlated with FEV_1 values (p<0.007). Our results suggest an autocrine role of FGF-FGFR-1 system in the pathogenesis of COPD-associated vascular remodeling.

2.2 Introduction

Chronic obstructive pulmonary disease (COPD) is a global health problem with increasing morbidity and mortality (1). One of the major causal factors is tobacco smoking (2). However, only ten percent of all smokers develop symptomatic COPD. The causes of this variability in response of the airways and lung parenchyma to tobacco smoke exposure have remained largely unclear. One of the key pathological features of COPD is thickening of airway walls as a result of inflammation, hyperplasia of airway smooth muscle cells and fibroblasts, and increased deposition of extracellular matrix (3). In addition, advanced COPD leads to pathological changes in the pulmonary circulation (4, 5). At least part of this is probably the result

of alveolar hypoxia, which is well known to cause pulmonary vasoconstriction and, if the hypoxic stimulus persists, pulmonary vascular remodeling, of which increased muscularization of small arterial branches is the most striking feature (6). With sustained vasoconstriction of pulmonary arteries, arterioles and veins, the medial vascular smooth muscle (VSM) extends distally to vessels normally devoid of smooth muscle (6). Intimal thickening due to fibrosis and emergence of smooth muscle cells within the intima of small pulmonary arterial branches has also been reported (5). Finally, loss of the pulmonary vascular bed by emphysema has been suggested to lead to the formation of new vessels (6). Thus, several phenomena acting in concert in COPD result in pulmonary vascular remodeling. Yet, little is known about the molecular mechanisms underlying these processes in the context of COPD.

A variety of growth factors and cytokines released from various sites of airway and vascular walls have the potential to contribute to the pathogenesis of vascular remodeling in COPD. In view of their important role in chronic inflammation, fibrosis and repair of various tissues, including the lung (9), fibroblast growth factors (FGFs) may well play a pivotal role in airway and vessel wall remodeling (7, 8). Fibroblast growth factors exert their biological effects via binding to four high-affinity, transmembrane tyrosine-kinase receptors designated FGFR-1 through FGFR-4 (9). Distinct FGF subtypes bind with different affinity to the various FGF receptors. Alternative splicing and regulated protein trafficking further modulate the intra-cellular events and resultant response initiated by FGF ligand-receptor interaction (9). In the lung as well as in the vascular system, FGFs have been implicated in several pathological conditions. FGF-1 and FGFR-1 were shown to be upregulated during the development of lung fibrosis (10). FGF-2 and also PDGF have been implicated in the pathogenesis of obliterative bronchiolitis after transplantation (11). Moreover, vascular remodeling in response to increased blood pressure is associated with elevated levels of basic fibroblast growth factor (12, 13).

To investigate, whether the FGF-FGFR system might be involved in the pathogenesis of COPD, we examined the expression patterns of FGF-1, FGF-2 and FGFR-1 in (ex-) smokers with or without COPD and correlated the expression with histological evidence of pulmonary vascular remodeling.

2.3 Materials and Methods

Selection of Patients' Specimens

We examined lung tissue specimens of subjects with or without COPD. Peripheral part of the lung tissue from current and ex-smokers who underwent lobectomy or pneumonectomy for lung cancer was obtained from the Pathology Laboratories of the Leiden University Medical Center, Leiden, the Netherlands, and the Zuiderziekenhuis, Rotterdam, the Netherlands. Tissue specimens were taken distally from the lung hilus part and contain predominantly parenchyma and small airways as well as vasculature. All lung tissues were inflated by an injection syringe using formalin and fixed for approximately 24 hours after which the tissues were further dehydrated and embedded in paraffin and subsequently processed for immunohistochemical staining. Based on a number of lung function data, patients were assigned to the COPD and non-COPD groups (14, 15).

COPD group. Fifteen subjects were assigned to the COPD group on the basis of the following parameters: forced expiratory volume in one second (FEV_1) <75% of predicted value (16) before bronchodilatation, FEV_1/FVC ratio <75%, a reversibility in FEV_1 ≤12% of predicted after 400 µg inhaled salbutamol, and a transfer factor for carbon monoxide (diffusion capacity) per liter alveolar volume (K_{co}) ≤80% of predicted value.

Non-COPD group. Thirteen subjects were assigned to the non-COPD group on the basis of the following data; a FEV_1 >85% before bronchodilatation, FEV_1/FVC ratio >85%, and reversibility in FEV_1 ≤12% of predicted after 400 µg salbutamol inhalation. In order to exclude accompanying lung disease leading to a restrictive function disorder, the total lung capacity (TLC) of each subject included in the study was over 80% of the predicted values (16).

Clinical data of all patients were examined for possible comorbidity and medication usage. All patients were free of symptoms of upper respiratory tract infection and none received antibiotics perioperatively. None of the patients received glucocorticosteroids in the three months prior to operation; four patients received oral glucocorticosteroids perioperatively. In addition to the rigorous criteria based on lung function parameters, microscopic exclusion criteria was also applied in the selection of patients for this study. After the selection based on lung function, all the lung tissues were subsequently examined histologically by two experienced lung

pathologists using following exclusion criteria: (i) presence of tumor in the lung tissue specimen submitted for this study, (ii) presence of poststenotic pneumonia in the specimen, (iii) fibrosis of lung tissue, and (iv) obstruction of the main bronchus of the resection specimen by tumor (14, 15).

Pulmonary Function Tests

All pulmonary function tests were performed within 3 months prior to surgery. FEV_1 and forced vital capacity (FVC) were measured by spirometry, total lung capacity and residual volume with the closed circuit helium dilution test and the K_{CO} using the single breath-holding technique, as described by Quanjer and co-workers (16). Lung function data and other patient characteristics are shown in Table 2.1.

Immunohistochemistry

Sections of paraffin-embedded lung tissue were cut at 4 μm, mounted on Super Frost Plus® microscopic slides (Menzel-Gläser, Braunschweig, Germany) and processed for immunohistochemistry. Serial sections were used for immunostaining of FGF-1, FGF-2 and FGFR-1 using human specific antibodies. The optimal dilutions for all antibodies were identified by examining the intensity of staining obtained with a series of dilutions, which gave specific and easily visible signal on paraffin sections derived from the same control tissue prior to perform the staining protocol on all section. In order to avoid day to day variations in the staining intensities, the incubations of all specimens with each antibody were performed in one single run. Sections were deparaffinized and rehydrated prior to incubation with specific mouse monoclonal and affinity purified antibodies against FGF-1 (1:2000 dilution), FGF-2 (1:200 dilution) and FGFR-1 (1:2000 dilution). The mouse IgG1 antibody against human FGF-1 was raised using a synthetic peptide corresponding to the internal 61-99 amino acid sequence whereas, the mouse IgG2b antibody was raised against a synthetic peptide corresponding to the 16 amino acids from the C-terminus of human FGFR-1, as described previously (17, 18). FGF-2 was a mouse (IgG1 isotype) monoclonal antibody raised against human FGF-2 (Mol. Weight: 18-24 Kda) and it was procured from Transduction laboratories, Lexington, Ky, USA. Anti-human mouse monoclonal antibodies against α-smooth muscle actin (α-SMA), Ki-67 and FGF-2 were purchased from NeoMarkers (Clone 1A4, Fremont, CA, USA), from Biogenex

(San Ramon, MO, USA) and from Transduction Laboratories (Lexington, USA), respectively. To block non-specific binding, sections were preincubated with 10% normal goat serum diluted in 5% bovine serum albumin in phosphate buffered saline (5% BSA/PBS, pH = 7.4). Subsequently, sections were incubated for overnight at 4 °C with the primary antibodies (FGF-1 and FGFR-1) diluted appropriately or for 1 hour at room temperature in case of α-SMA (1:1000 dilution). Secondary biotinylated anti-immunoglobulins (Multilink®, 1:75 dilution, Biogenex, San Ramon, MO, USA) and tertiary streptavidin conjugated Alkaline Phosphatase (Label® 1:50 dilution, Biogenex, San Ramon, MO, USA) were used to enhance the detection sensitivity. Color was developed using New Fuchsin, while endogenous alkaline phosphatase activity was inhibited by 0.01 M levamisole.

FGF-2 and Ki-67 immunostaining was performed on serial sections after antigen retrieval by boiling in citrate buffer (10 mM citrate buffer, pH = 6.0) for 10 minutes in a microwave oven. Sections were preincubated with 10% normal goat serum in 5% BSA/PBS, followed by incubation with primary antibody (1:50 dilution) overnight at 4 °C. Slides were rinsed in PBS, incubated for 30 minutes with peroxidase-conjugated streptavidin at a dilution of 1:50 (Biogenex, San Ramon, MO, USA). Subsequently, sections were colored using 0.025% of 3,3-diaminobenzidine (Sigma, St Louis, MO, USA) in 0.01 mol/L PBS, containing 0.03% H_2O_2. Slides were counterstained with Mayer's hematoxylin. Positive controls consisted of human breast carcinoma and placental tissue. The optimal dilutions for all antibodies were identified by examining the intensity of staining obtained with a series of dilutions, which gave specific and easily visible signal on paraffin sections derived from the same control tissue. Slides were mounted and staining results were systematically investigated (see below). Negative controls consisted of omission of the primary antibody.

Semi-quantitative Analysis

All tissues were analyzed in a blinded fashion in random order by two independent observers, who were unaware of the clinical data of the case under study. Semi-quantitative analysis was performed using an arbitrary visual scale with grading scores of 0, 1, 2, and 3 representing no, weak, moderate and intense staining, respectively (14, 15). Errors within and between observers were assessed by correlating the expression scores using Pearson's analysis and we found a very high

correlation ranging from 0.8 to 0.9. Microphotographs in Figure 2.1, panel *A* to *D* show representative examples of staining intensities used for visual scoring, 0-3 respectively. Sections were graded for the intensity of expression signal of FGF-1, FGF-2 and FGFR-1 in the endothelium and VSM of small (50-200 µm internal diameter) and in the endothelium, VSM and adventitial area of large (>200 µm internal diameter) pulmonary arteries.

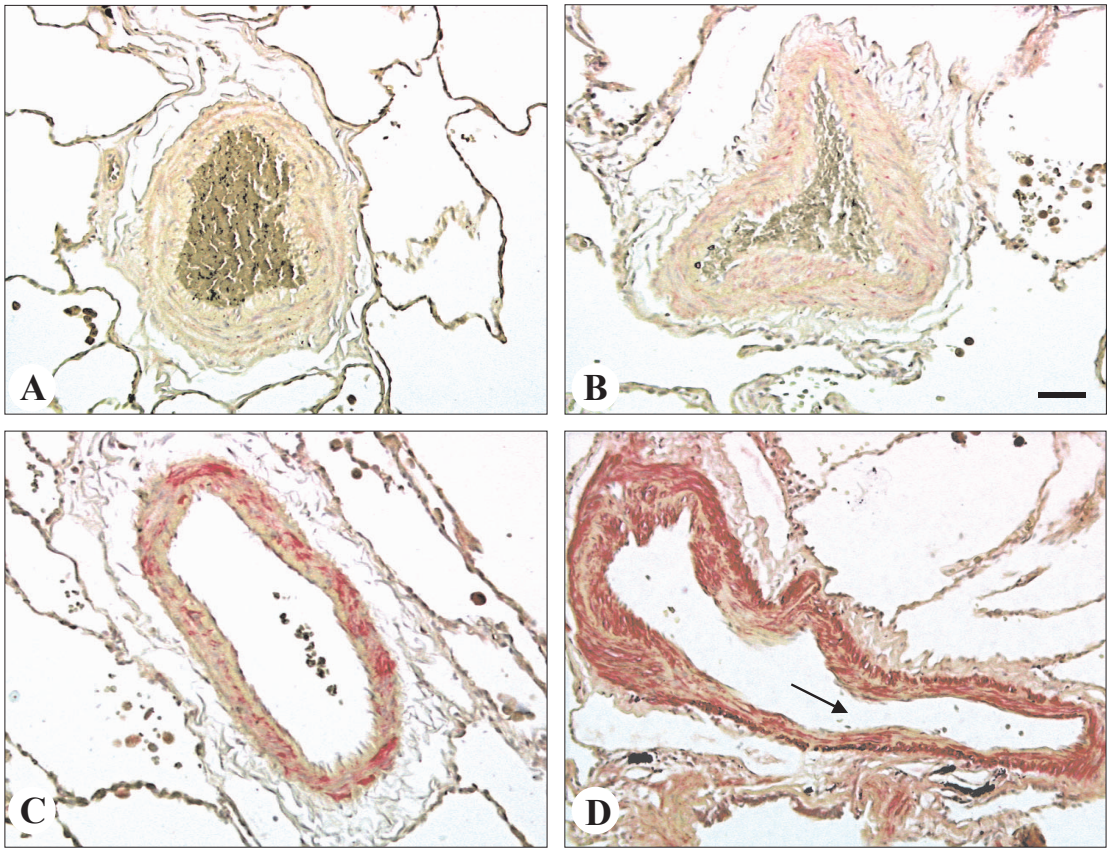

Figure 2.1 **Representative examples of staining intensity pattern used for visual scoring.** Photomicrographs depict lung tissue sections from patients without COPD (***A*** and ***C***) and with COPD (***B*** and ***D***) showing FGFR-1 staining (red new-fuchsine) in vascular smooth muscle cells. Panels ***A*** to ***D*** show representative examples of staining intensities used for visual scoring, 0-3 respectively. Scale bar = 50 µm; original magnification: x100.

Video Image Analysis

In addition, video image analysis was performed for α-SMA staining using Leica Qwin system version 3.0 (Leica B.V., Rijswijk, The Netherlands). Twenty digital images (pixel size: 736x574) from each section were taken using a video camera. Internal diameter of blood vessels was derived as a mean of measured vertical and

horizontal diameters. In our study, we excluded those vessels who showed the ratio of >3 for both the diameters. Based on the internal diameter, pulmonary vessels were grouped into 4 sizes (50-100 μm, 100-200 μm, 200-400 μm and >400 μm). Vascular wall (VW) area, α-smooth muscle actin (α-SMA) stained area and vessel internal diameter (ID) were measured. Measurements were expressed as percentages for staining per vessel wall (α-SMA/VW area), for VW area corrected for internal diameter (VW area/ID) and α-SMA staining, also corrected for lumen diameter (α-SMA area/ID).

Statistical Analysis

Data were analyzed for statistical significance using the unpaired, two-tailed Students' "t"-test as well as the Mann-Whitney non-parametric test, wherever appropriate (14,15, SPSS software packet-SPSS Incorporation, Chicago, USA). The staining score data for FGF-1, FGF-2 and FGFR-1 were expressed as mean ± SEM. Furthermore, FGF-1, FGF-2 and FGFR-1 staining scores for different vessels with internal diameter > and <200 μm, were correlated with FEV_1 using Pearson's correlation analysis. Furthermore, the individual FEV_1 values were correlated with the vascular remodeling data (VW area/ID) in both the groups. Differences with $p \leq 0.05$ were considered to be statistically significant.

2.4 Results

Clinical Parameters

The clinical and lung function characteristics of all subjects included in the study are listed in the Table 2.1. FEV_1 and FEV_1/FVC values were significantly lower in the COPD group than in the non-COPD group ($p < 0.001$). In the COPD group, residual volume (RV) was increased, whereas CO-diffusion (K_{co}) was reduced ($p < 0.005$). The subjects in the two groups did not differ significantly with respect to age, total lung capacity (TLC), reversibility in FEV_1, smoking status (pack-years) and previous steroid usage (Table 2.1).

Table 2.1 Subject characteristics and clinical parameters

	Non-COPD	COPD
FEV_1	99±1.9	53±3.2*
$dFEV_1$	3±0.6	4±0.9
FEV1/FVC	100±2.3	58±5.0*
TLC	104±2.0	108±8.8
RV	115±5.5	141±15.4*
Kco	94±2.0	55±5.4*
Sex (Male/Female)	11/2	14/1
Age (years)	57±3.2	59±5.0
Smokers/ex-smokers/non-smokers	9/4/0	12/3/0
Pack-years	33±4.7	35±5.2
Steroid use (yes/no/unknown)	0/12/1	4/9/2

Abbreviations: Forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), total lung capacity (TLC), residual volume (RV), reversibility of FEV_1 after 400 μg salbutamol ($dFEV_1$) and carbon monoxide diffusion constant (Kco) are given as percentage of predicted. FEV_1/FVC is given as actual ratio in %. * $P < 0.005$ versus non-COPD.

Localization and quantification of FGF-1 and FGF-2

FGF-1 expression was detected in the media and adventitia of large pulmonary arteries and veins, but only in the media of small vessels. No FGF-1 staining was found in the endothelial layer of any vessels. FGF-2 was localized specifically in nuclei of endothelial and vascular smooth muscle cells. Expression of FGF-1 and FGF-2 was also observed in epithelial and bronchiolar smooth muscle cells. Representative microphotographs showing the expression patterns of FGF-1 and FGF-2 are presented in Figure 2.2. A summary of the semi-quantitative data of FGF-1 and FGF-2 immunostaining is given in Figure 2.3. In subjects with COPD we observed significantly increased ($p<0.02$) expression of FGF-1 in medial VSM of larger vessels, but the level of adventitial expression of FGF-1 remained unaltered (Figure 2.3, panel A). In contrast to FGF-1, the expression of FGF-2 was elevated in the COPD group, but only in the small vessels with an internal diameter <200 μm, where it was increased in endothelial ($p<0.007$) and medial smooth muscle ($p<0.001$) cells (Figure 2.3B).

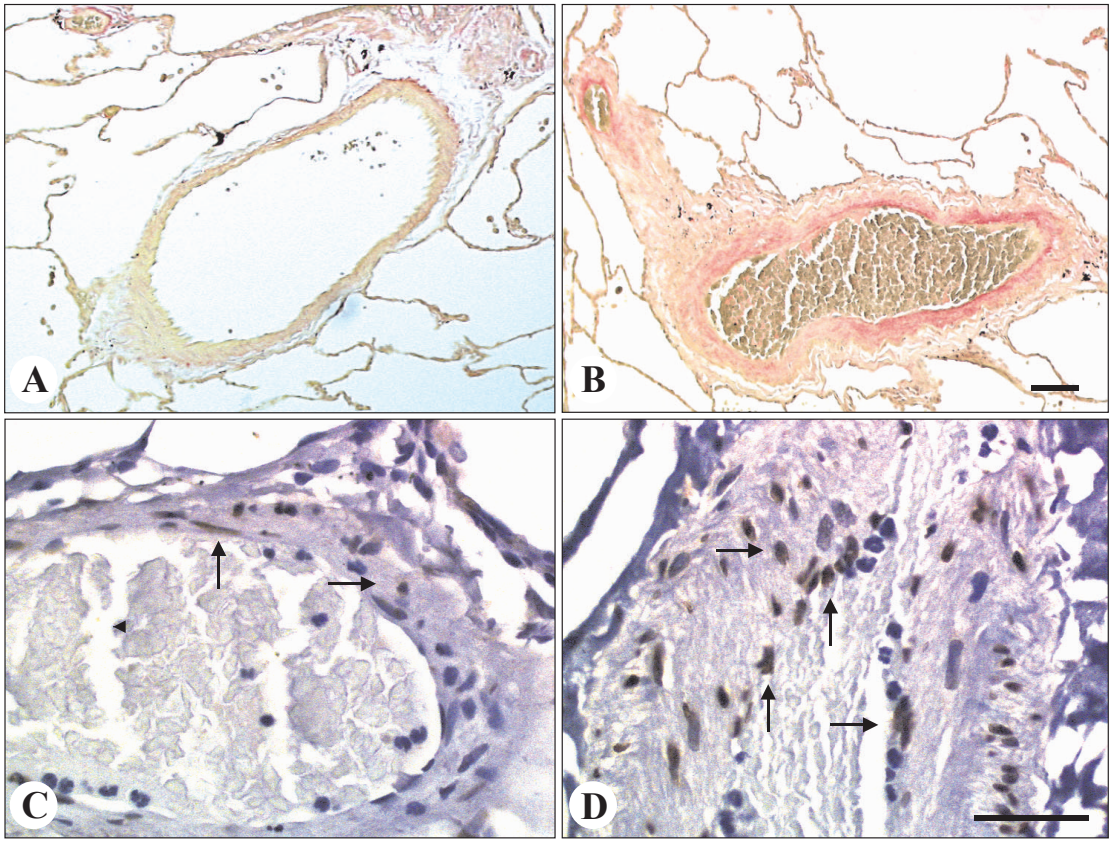

Figure 2.2 **Photomicrographs of lung tissue sections from patients without COPD (*A* and *C*) and with COPD (*B* and *D*).** Panels ***A*** and ***B*** (scale bar = 50 µm; original magnification: x100) show representative examples of FGF-1 protein staining (red new-fuchsine) in vascular smooth muscle cells of a large vessel (internal diameter >200 µm). Panels ***C*** and ***D*** (scale bar = 100 µm; original magnification: ×400) show representative examples of nuclear FGF-2 expression (brown 3,3-diaminobenzidine) in endothelium and vascular smooth muscle cells of vessels with internal diameter <200 µm. Arrows indicate positive nuclei.

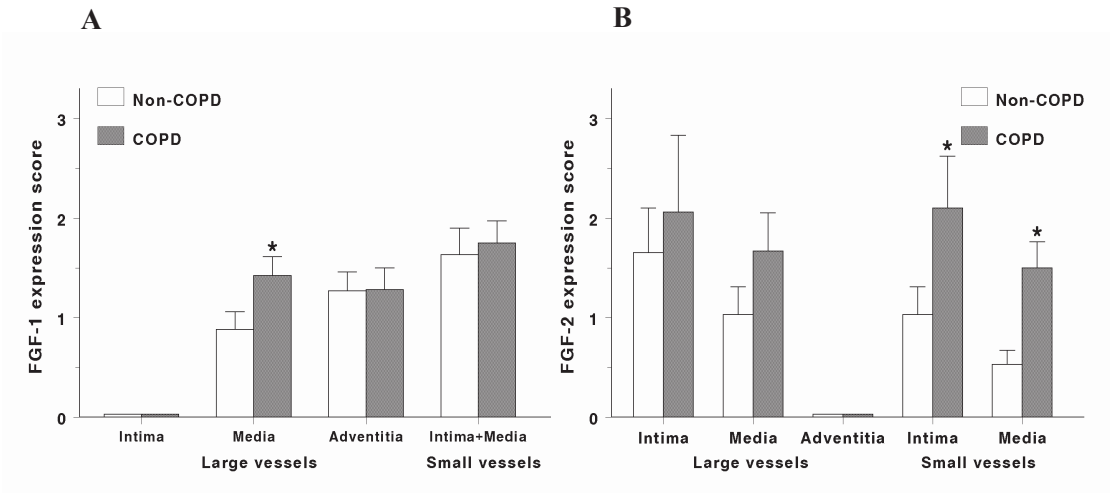

Figure 2.3 Graphic representations of FGF-1 (***panel A***) and FGF-2 (***panel B***) expression scores (mean ± SEM) in large (internal diameter >200 µm) and small (internal diameter <200 µm) vessels in non-COPD and COPD groups. * ***P*** <0.05 versus the non-COPD group.

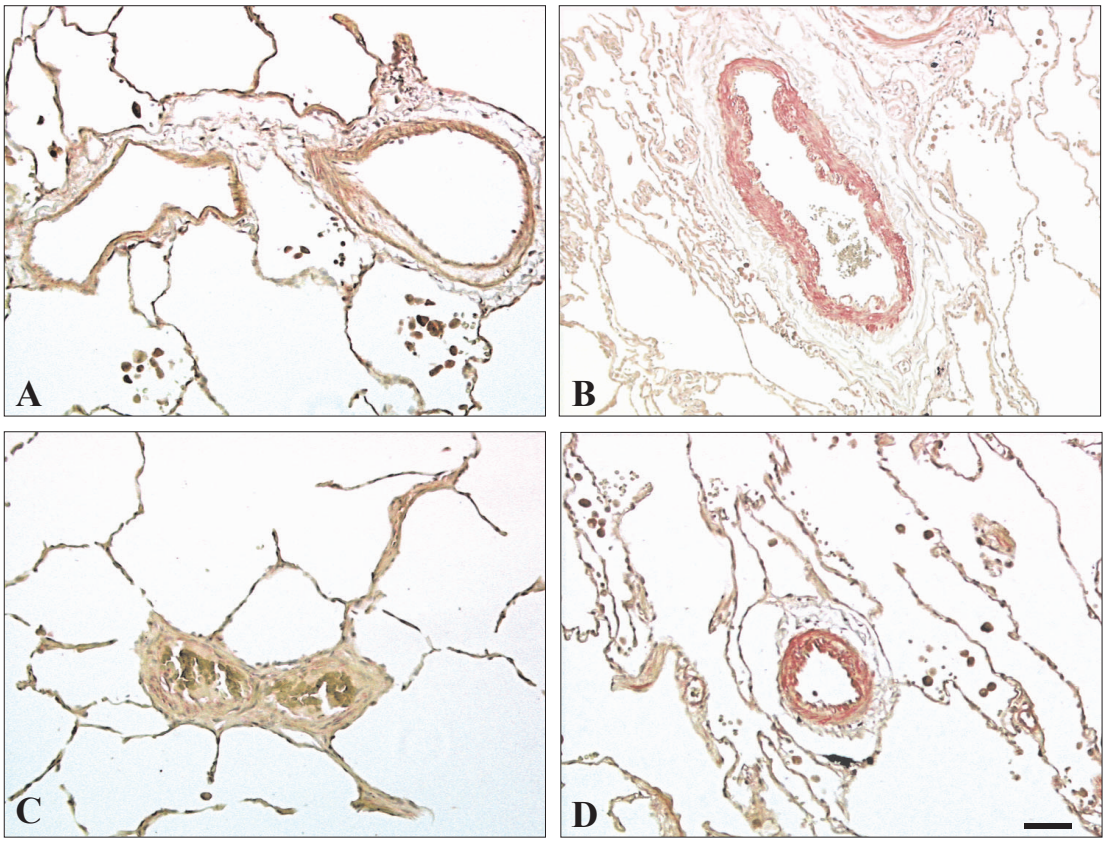

Figure 2.4 Photomicrographs of lung tissue sections from patients without COPD (***A*** and ***C***) and with COPD (***B*** and ***D***) showing FGFR-1 staining (red new-fuchsine) in vascular smooth muscle cells from large (internal diameter >200 µm; ***A*** and ***B***) and small (internal diameter <200 µm; ***C*** and ***D***) blood vessels. Scale bar = 50 µm; original magnification: x100.

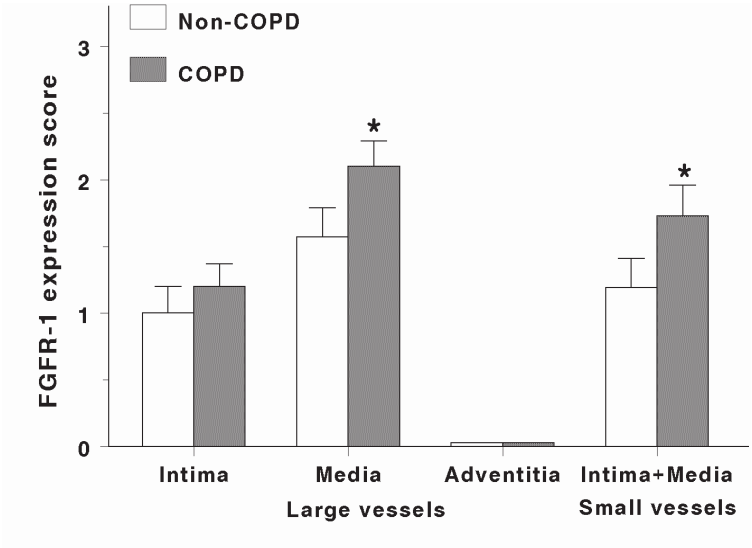

Figure 2.5 Graphic representations of FGFR-1 expression scores (mean ± SEM) in large (internal diameter >200 µm) and small (internal diameter <200 µm) vessels in non-COPD and COPD groups. * ***P*** <0.05 versus the non-COPD group.

Localization and quantification of FGFR-1

FGFR-1 immunoreactivity was detected in epithelial and bronchiolar smooth muscle cells, and in the endothelium and vascular smooth muscle of large and small vessels. No adventitial positivity for FGFR-1 was observed. Representative microphotographs showing the expression pattern of FGFR-1 are presented in Figure 2.4. A graphic representation of the data of FGFR-1 immunostaining is given in Figure 2.5. The expression of FGFR-1 was significantly elevated in medial smooth muscle cells of large vessels ($p<0.04$) in the COPD-group as compared to non-COPD group, whereas the staining for the receptor in the intimal endothelium remained unaltered. Moreover, in contrast to the FGF-1 expression in small vessels in COPD patients, we found significantly higher expression levels of the receptor ($p<0.05$) in medial smooth muscle of small vessels (Figure 2.5).

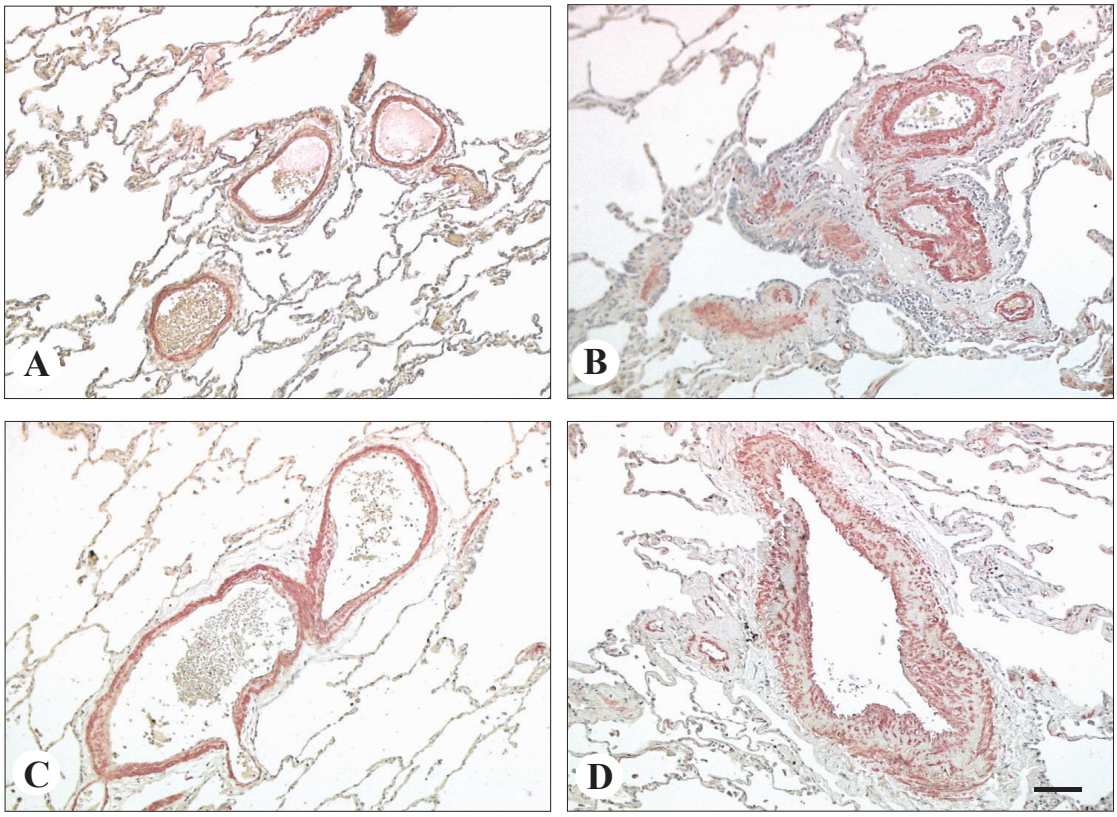

Figure 2.6 Photomicrographs of lung tissue sections from patients without COPD (***A*** and ***C***) and with COPD (***B*** and ***D***) showing α-smooth muscle actin staining (red new-fuchsine) in vascular smooth muscle cells from small (internal diameter <200 µm; ***A*** and ***B***) and large (internal diameter >200 µm; ***C*** and ***D***) blood vessels. Scale bar = 50 µm; original magnification: x100).

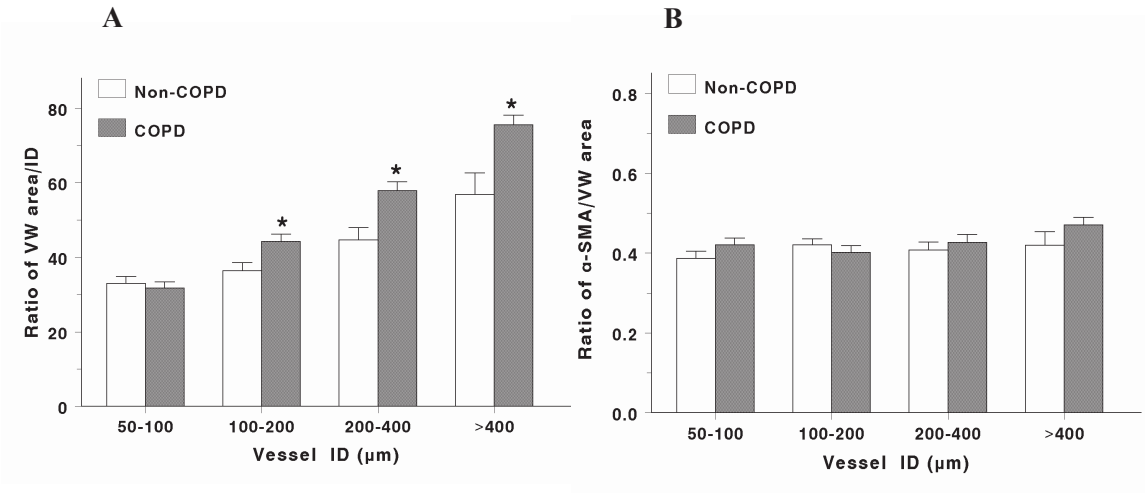

Figure 2.7 **Graphic representations of vessel wall measurements (mean ± SEM) using video image analysis in non-COPD and COPD groups.** Panel ***A***: Ratio of vascular wall area/internal diameter (VW area/ID) ratio. Panel ***B***: Ratio of α-SMA area/VW area. * ***P*** <0.05 versus the non-COPD group.

Assessment of vascular remodeling

To examine pulmonary vascular remodeling as evidenced from variations in wall thickness and muscular medial thickness, video image analysis was performed using α-SMA immunostaining. Four separate groups with vessels of internal diameters of 50-100, 100-200, 200-400 and >400 μm, respectively, were analyzed (Figure 2.6). Measurements (mean ± SEM) were expressed as VW area/ID, α-SMA area/ID or percentage α-SMA staining per vessel wall area that represents volume fraction for smooth muscle staining was shown as α-SMA/VW area. The graphic representation of vascular wall remodeling data is presented in Figure 2.7. A significant increase in VW area/ID ratio for COPD in vessels of 100-200 μm (44.2 ± 1.9 vs. 36.4 ± 2.1, p=0.007), 200-400 μm (57.9 ± 2.4 vs. 44.7± 3.2, p<0.001) and >400 μm (75.6 ± 2.6 vs. 56.8 ± 5.9, p=0.011) was found (Figure 2.7, panel A). In vessels ranging from 50 to 100 μm in internal diameter no differences in VW area/ID ratio were observed. A significantly increased α-SMA area/ID ratio was observed for COPD in the 200-400 (26.7 ± 1.9 vs. 20.3 ± 2.9, p = 0.034) and >400 μm (38.3 ± 2.0 vs. 23.5 ± 3.4, p=0.006) internal diameter vessels but not in the 50-100 and 100-200 μm vessels. Surprisingly, no significant differences were observed between COPD and non-COPD groups in the percentage vascular smooth muscle, defined as α-SMA/VW area in all vessel types (Figure 2.7, panel B). Proliferation of VSM cells as evidenced from Ki-67 positivity was observed only very occasionally (data not shown).

Correlation with clinical data

The staining scores of FGF-1, FGF-2 and FGFR-1 expression in COPD and non-COPD patients were analyzed using Pearson's test. For FGF-1, we observed a weak but significant inverse correlation (r=-0.39, p=0.038) between staining score and FEV_1 in the medial VSM of vessels > 200 μm in ID (Figure 2.8, panel A). Additionally, there was a significant inverse correlation of FGF-2 staining scores in both endothelium (r=-0.44, p=0.002) and medial VSM (r = -0.55, p<0.0001) of vessels <200 μm in internal diameter with FEV_1 (Figure 2.8, panel B and C). However, in vessels >200 μm in internal diameter, no significant correlation between FGF-2 expression and FEV_1 was found (data not shown). Surprisingly, staining scores for FGFR-1 were not significantly correlated with FEV_1 (data not shown). When considering the association between FEV_1 and medial hypertrophy (VW area/ID ratio), we observed a significant inverse correlation of -0.50 (p=0.007) for vessels with internal diameter >200 μm (Figure 2.8, panel D). However, no significant correlation could be established between FEV_1 and VW area/ID ratio for the vessels with internal diameter <200 μm (r = -0.10, p>0.10).

2.5 Discussion

In this study we have found that COPD is associated with an increase in the expression of FGF-2 in small (<200 μm) and FGF-1 in large (>200 μm) pulmonary vessels respectively whereas, FGFR-1 is increased in both vessel types. Vascular medial thickness, assessed by video image analysis, was significantly increased in COPD in pulmonary vessels of various sizes. Pearson's correlation analysis revealed a significant inverse correlation of FEV_1 with FGF-1 staining in the media of large and with FGF-2 expression in both endothelium and VSM of small vessels. Additionally, an inverse correlation of FEV_1 with medial thickening was found in pulmonary vessels of larger caliber, indicating that the degree of pulmonary vascular remodeling is related to the severity of obstructive lung function defect.

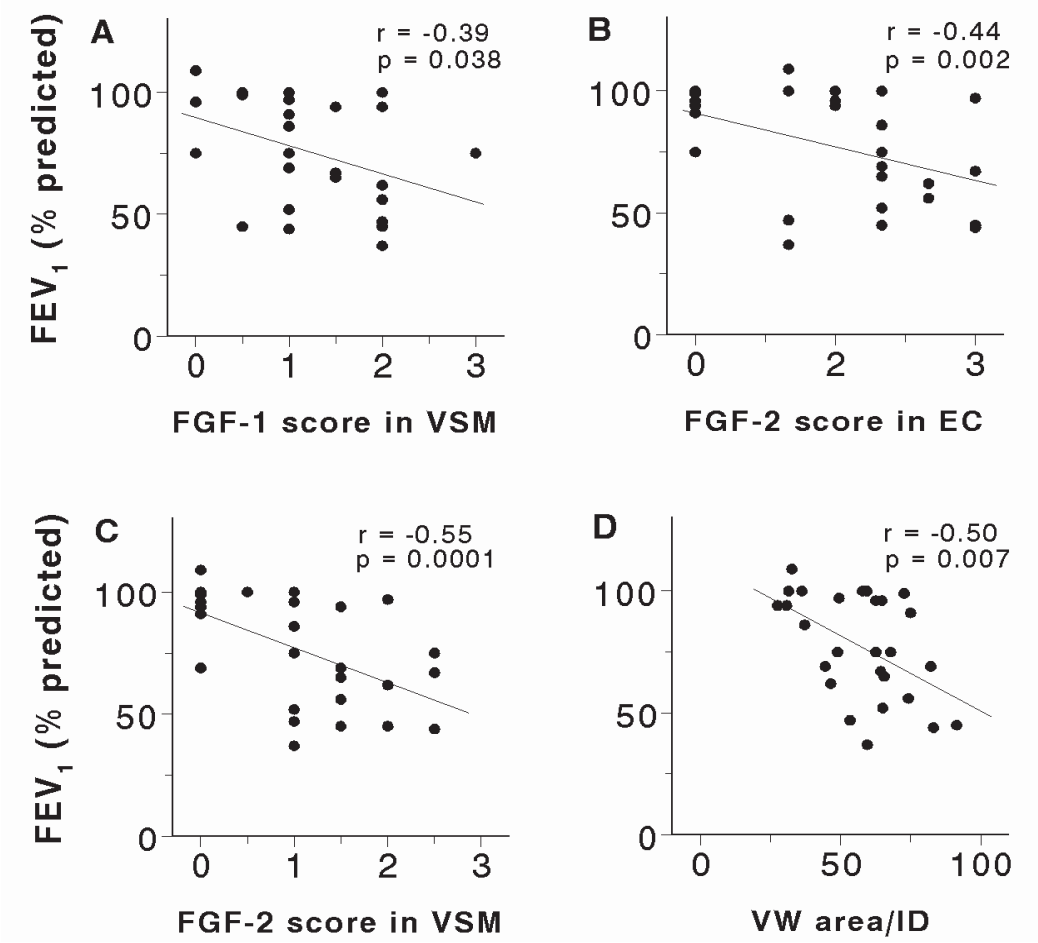

Figure 2.8 **Correlation with FEV_1 (% predicted) of FGF-1 expression in vascular smooth muscle cells** (internal diameter >200 μm) (***A***), FGF-2 expression in vascular smooth muscle cells (internal diameter <200 μm; ***B***), FGF-2 expression in endothelial cells (EC) from small blood vessels (internal diameter <200 μm; ***C***) and vascular wall area/internal diameter (VW area/ID; ***D***). Correlation coefficient (r) and significance level (***P*** value) were obtained using linear regression (Pearson's) analysis.

Several studies have commented on the importance of structural and functional abnormalities in the pulmonary vasculature of COPD patients. Hypoxia is known to induce prompt and severe vasoconstriction in the pulmonary vasculature, and sustained lung tissue hypoxia, as results from obstructive lung disease such as COPD, leads to pulmonary hypertension (4, 19). Hypoxic vasoconstriction is considered to represent one of the major contributing factors of pulmonary hypertension and right-sided heart failure in COPD and other chronic pulmonary diseases (4, 19). In addition, emphysema, accompanied by loss of elastic recoil, increased pulmonary pressure and destruction of part of the pulmonary microvasculature, may contribute to the increased vascular resistance observed in COPD (5, 6). Using Video image analysis, we assessed systematically vascular wall thickening in COPD patients and

non-COPD cases. Wall thickness of vessels 200 μm or more in diameter was increased in COPD. Our results on pulmonary vascular remodeling particularly in terms of intimal and medial thickening are in agreement with several earlier reports (4, 5, 20-22). Furthermore, the degree of intimal and medial thickening correlated with the decrease in lung function and, hence, with the severity of the disease. Wright and coworkers (4, 5) also observed a correlation with the severity of disease with mild to moderate COPD with intimal thickening and in severe cases with medial thickening. Similar findings on vascular abnormalities in COPD were recently reported by Peinado and coworkers, who showed intimal but not medial thickening in the vasculature of mild COPD patients compared to non-smoking controls (20, 21).

We used expression of smooth muscle marker α-SMA (23) to investigate whether the ratio of smooth muscle (α-SMA/VW area) in the vascular wall had changed during the progression of COPD. Surprisingly, the ratio of α-SMA stained area to VW area remained unchanged. Approximately 42% of cells in all vessels stained positive for α-SMA, indicating that the increase in wall thickness could be attributed to the deposition of extracellular matrix proteins and medial accumulation of other cells, such as inflammatory cells and fibroblasts. Recently, we found specific staining for extracellular matrix proteins, like fibronectin and collagen subtypes in the intimal vascular cells of these pulmonary vessels indicating for ongoing intimal fibrosis in COPD patients (data not shown). Taken together, the data from this study indicate that vascular remodeling in COPD could be a contributing event in the pathogenesis of pulmonary hypertension in these patients. Furthermore, the observed changes in the intimal fibrosis as well as medial thickening could narrow the vessel caliber and may eventually lead to more severe vascular obstruction in COPD patients.

Members of the fibroblast growth factor family FGF-1, FGF-2 and FGFR-1 are constitutively expressed in normal human lungs, particularly in airway epithelium, monocytes, and are localized in the intima and media of pulmonary vessels (24). Pulmonary expression patterns of FGF-1, FGF-2 and FGFR-1, as found in our study are in agreement with results obtained by Hughes and Hall (24) in the normal lungs. However, in the peripheral regions of the lungs of patients with COPD, we observed additionally FGF-1 in adventitia and FGF-2 immunoreactivity in the nuclei of medial

smooth muscle and endothelial cells advocating for a potential role of FGF-FGFR system in vascular remodeling in COPD.

Fibroblast growth factor family members are implicated in tissue remodeling in a wide variety of pathophysiological conditions including systemic hypertension, ischemic heart disease and interstitial lung fibrosis (10, 12, 25, 26). Barrios and coworkers (10) showed FGF-1 and FGFR-1 expression in experimentally induced pulmonary fibrosis. Becerril and colleagues found that FGF-1 expression in the lung fibroblasts results in down-regulation of collagen synthesis and up-regulation of collagenases, which may protect against fibrosis (27). However, increased FGF-2 and FGFR-1 expression in vascular smooth muscle cells *in vitro* in response to vascular injury has been shown to be associated with extracellular matrix remodeling, cellular proliferation, down-regulation of collagen type I and up-regulation of collagenase MMP-1 (28). Our findings of upregulated FGF-1, FGF-2 and FGFR-1 expression could indicate that such compensatory mechanisms are active in COPD since smoking has been suggested to affect cellular viability in lungs.

In a recent study, Singh and colleagues demonstrated that increased nuclear expression of FGF-2 in vascular smooth muscle and endothelium precedes arterial enlargement in response to increased arterial blood flow *in vivo* (12). Though the function of high molecular weight FGF-2 in the cell nucleus remains unclear, it is believed that this form of FGF-2 translocates to the nucleus. Moreover, Stachowiak and coworkers have demonstrated co-localization of the receptor FGFR-1 and FGF-2 in the nucleus of human astrocytes suggesting for novel mechanisms for the action of FGF-2 (29). In this study we show that FGF-2 is localized in the nucleus of endothelial and VSM cells and that the expression is increased in pulmonary vessels with diameter >200 μm in patients with COPD indicating a role for this growth factor in vascular remodeling. We also showed that in COPD, the expression of FGF-2 was upregulated in vessels with an internal diameter of >200 μm. Bryant et al. (13) recently found that administration of FGF-2 could inhibit internal luminal area decrease and wall thickening in response to altered blood flow; furthermore, this inhibitory effect could be blocked by anti-FGF-2 neutralizing antibodies. Our findings suggest that FGF-2 plays an important role in the response to increased pressure in the pulmonary vasculature in COPD. Several studies on hypoxia-induced pulmonary hypertension have shown that increased smooth muscle mass develops as a result of hypertrophy and hyperplasia of pre-existing smooth muscle cells or results

from differentiation of fibroblasts recruited to the media from the adventitia (23, 30). It is likely that such mechanisms of vascular smooth muscle mass increase are operational in COPD-related vascular remodeling.

In vivo and *in vitro* data indicate that smooth muscle cells, and their cross-talk with endothelium, myofibroblasts and inflammatory cells via growth factors and cytokines, are major contributing factors to vascular remodeling during different pathophysiological conditions (23, 27, 31, 32). Furthermore, inflammation, a well-established factor in peripheral as well as in central airways in COPD, could also be associated with vascular remodeling in COPD. Increased adventitial infiltration of inflammatory cells, predominantly $CD8^+$ T-lymphocytes, in muscular pulmonary and bronchiolar arteries has been reported earlier (21, 33). Taken together, our results support the notion that in COPD, increased vascular expression of FGF-1, FGF-2 and FGFR-1 could participate in an autocrine and/or in a complex growth factor-cytokine interactive manner in regulating the process of pulmonary vascular remodeling. Our data further support the hypothesis that COPD is associated with pulmonary vascular remodeling and that the FGF-FGFR system contributes to the pathogenesis and severity of the disease.

Acknowledgements

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Chapter 3

Vascular Endothelial Growth Factor and its Receptors in COPD

Adapted from:

Kranenburg A. R., de Boer W. I., Alagappan V. K. T., Sterk P. J. and Sharma H. S.: Enhanced Bronchial Expression of Vascular Endothelial Growth Factor and Receptors (Flk-1 and Flt-1) in Patients with Chronic Obstructive Pulmonary Disease. *Thorax* - In press.

3.1 Summary

Background: Ongoing inflammatory processes resulting in airway and vascular remodeling characterize chronic obstructive pulmonary disease (COPD). Vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) could play a role in tissue remodeling and angiogenesis in COPD.

Methods: We examined the cellular expression pattern of VEGF, Flt-1 and KDR/Flk-1 by immunohistochemistry in central and peripheral lung tissues obtained from (ex-) smokers with (FEV_1 <75% predicted; n=14) or without COPD (FEV_1 >85% predicted; n=14). The immunohistochemical staining of each molecule was quantified using a visual scoring method with grades ranging from 0 (no), 1 (weak), 2 (moderate) to 3 (intense).

Results: VEGF, Flt-1 and KDR/Flk-1 immunostaining was localized in vascular and airway smooth muscle (VSM and ASM) cells, bronchial, bronchiolar and alveolar epithelium and macrophages. Pulmonary endothelial cells abundantly expressed Flt-1 and KDR/Flk-1 but not VEGF. In COPD patients, bronchial VEGF expression was higher in microvascular VSM cells and ASM cells as compared to non-COPD patients (1.7 and 1.6 fold, $p<0.01$, respectively). VEGF expression in intimal and medial VSM (1.7 and 1.3 fold, $p<0.05$) of peripheral pulmonary arteries associated with the bronchiolar airways was more intense in COPD, as well as in small pulmonary vessels in the alveolar region (1.5 and 1.7 fold, $p<0.02$). In COPD patients, KDR/Flk-1 expression was enhanced in endothelial cells, intimal and medial VSM (1.3, 1.9 and 1.5 fold, $p<0.02$), whereas endothelial Flt-1 expression was 1.7 times higher ($p<0.03$). Furthermore, VEGF expression was significantly increased in bronchiolar and alveolar epithelium as well as bronchiolar macrophages (1.5 fold, $p<0.001$). Additionally, expression of VEGF in bronchial VSM and mucosal microvessels as well as bronchiolar epithelium inversely correlated with FEV1 ($r < -0.45$; $p<0.01$).

Conclusions: Our results suggest that VEGF and its receptors Flt-1 and KDR/Flk-1 are involved in peripheral vascular and airway remodeling processes in an autocrine and/or paracrine manner. This system may also be associated with epithelial cell viability during airway wall remodeling in COPD.

3.2 Introduction

Chronic obstructive pulmonary disease (COPD) is a disease state characterized by airflow limitation that is not fully reversible, usually progressive and associated with an abnormal inflammatory response of the lungs in response to noxious particles and gases (1). COPD is a major health problem with cigarette smoking as its main cause. One important pathological features of COPD is chronic airway inflammation characterized by an influx of inflammatory cells predominantly neutrophils, macrophages and $CD8^+$ T-lymphocytes in the lumen and wall of bronchial and bronchiolar airways and parenchyma (2-4). Furthermore, several studies reported a thickened bronchiolar wall and airway remodeling with peribronchiolar fibrosis, an increase in airway smooth muscle (ASM) mass and emphysema (3, 5, 6).

Vascular abnormalities have been associated with the development of COPD (7, 8). Wright *et al.* found an increase in wall area of small (< 500 μm) pulmonary vessels, by intimal thickening in mild to moderate COPD patients and medial thickening in severe cases as well, which was correlated with a decline in FEV_1 (7, 9). Furthermore, recent observations indicated that muscular pulmonary and bronchiolar arteries have increased adventitial infiltration of $CD8^+$ T-lymphocytes and have intimal thickening that was correlated to the amount of total collagen deposition (8, 10). Finally, emphysema may lead to loss of the pulmonary vascular bed and induce angiogenesis (11). Yet, little is known about the molecular mechanisms underlying these processes in the context of COPD.

One of the potent proteins involved in vascular remodeling is vascular endothelial growth factor (VEGF). The VEGF family currently comprises six members (VEGF-A to F), of which the originally identified VEGF-A_{165} variant is the predominant form of five additional spliced variants (12). VEGFs are heparin-binding proteins and act via their high affinity, transmembrane receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1). The receptors belong to the family of tyrosine kinases and are predominantly expressed by endothelial and epithelial cells (12). VEGF promotes an array of responses in the endothelium including hyperpermeability, endothelial cell proliferation and angiogenesis with new vessel tube formation in vivo (12, 13). The expression of VEGF can be induced under a variety of pathophysiological conditions, including pulmonary hypoxia and pulmonary hypertension with increased sheer stress

(13, 14). Both hypoxia and pulmonary hypertension are pathological features often seen in advanced COPD patients (2). We hypothesize, that increased VEGF expression perhaps under an influence of hypoxia-inducible transcription factors (HIFs) may contribute to increased and abnormal proliferation of endothelial and VSM cells in pulmonary vessels leading to vascular remodeling.

Although the role of VEGF in the vascular biology is thoroughly studied, it has become clear that VEGF and its receptor system are involved in various other cellular events as well, including epithelial proliferation and survival, and the recruitment of mast cells, neutrophils and macrophages to sites of fibrosis (13, 15, 16). Recent studies indicate that VEGF is expressed in the lung by bronchiolar, submucosal glandular and alveolar type I and II epithelial cells, alveolar macrophages, airway and vascular smooth muscle (ASM and VSM) cells as well as myo-fibroblast in fibrotic lung lesions (14, 17, 18).

In order to assess the role of VEGF and its receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) in the pathophysiology of COPD, we first examined the expression of VEGF-A, Flt-1 and KDR/Flk-1 in central and peripheral lung tissue from (ex-) smokers with or without COPD. Furthermore, we investigated the relation of lung function with the expression data of VEGF and its receptors.

3.3 Materials and methods

Selection of patients

Central and peripheral lung tissues were obtained from current or ex-smokers who underwent lobectomy or pneumonectomy for lung cancer. Fourteen subjects with COPD (FEV_1 < 75% predicted) and fourteen subjects without COPD (FEV_1 > 84% predicted) were included as previously described (19-21). Total lung capacities (TLC) were not below normal levels (TLC >80% predicted). All patients lack upper respiratory tract infection and did not receive antibiotics perioperatively. None of the patients had received glucocorticosteroids during 3 months period before resection, but four patients received glucocorticosteroids perioperatively. Based on these criteria, subjects with COPD could not be subdivided into patients with either chronic bronchitis or emphysema alone. Clinical data are given in Table 3.1. Subjects were excluded if the obstruction of the central bronchi was due to the tumor, or if diffuse pulmonary

inflammation or fibrosis was present, or if no tissue free from tumor could be obtained. Lung tissue specimens used in this study were obtained from the archival collection at the Department of Pathology (LUMC, Leiden, NL). Medical Ethics Committee of LUMC approved the study. The patients in these two groups participated in a larger research project, part of which has been published previously (19-21). Lung tissue specimens were routinely fixed in 10% neutral buffered formalin by inflation-immersion fixation and embedded in paraffin for histopathological examination and immunohistochemistry.

Immunohistochemistry

Paraffin sections (4 μm thick) of the lung tissues were cut and mounted on silane-coated glass slides. Immunohistochemistry was performed using a method as described earlier (20, 22, 23). In brief, after deparaffinization in xylene and rehydration through graded alcohol, slides were rinsed with phosphate buffered saline (PBS). Endogenous peroxidase was blocked with 0.3% hydrogen peroxidase. For VEGF, VEGFR-1, VEGFR-2 and Ki-67 staining, slides were pre-treated by boiling in citrate buffer (10 mM citrate buffer, pH = 6.0) for 10 minutes in a microwave oven. Subsequently, sections were preincubated with 10% normal goat serum diluted in 5% bovine serum albumin in phosphate buffered saline (5% BSA/PBS, pH = 7.4), and afterwards incubated for 30 minutes at room temperature with affinity-purified rabbit polyclonal VEGF antibody in a dilution of 1:200 v/v. The VEGF antibody used was raised against a 20 amino acid synthetic peptide corresponding to residues 1-20 of the amino terminus of human VEGF (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). A different series of slides were incubated with a rabbit polyclonal antibody against a synthetic peptide corresponding to aa 1312-1328 of human Flt-1 (NeoMarkers, RB-1526, Fremont, CA, USA) in a dilution of 1:100 v/v. For VEGFR-2, a rabbit polyclonal antibody against aa 1326-1345 of mouse KDR/Flk-1 (NeoMarkers, RB-1527, Fremont, CA, USA) in a dilution of 1:200 v/v was used. To examine proliferation of cells in the airways, an antibody against Ki-67 (Dako Corporation, Glostrup, Denmark) of 1:400 v/v at 4^{o}C overnight for was used as a marker. Consecutive tissue sections were also stained with a monoclonal mouse anti-human alpha-smooth muscle actin (α-SMA) antibody (clone 1A4: Biogenex, San Ramon, USA) in a dilution of 1:1000 v/v. The optimal dilution of the first antibody was identified by examining the intensity of

staining obtained with a series of dilutions of the antibody from 1:50 to 1:1000. Negative controls were prepared by omission of the primary antibody. After washing with tris-base buffered saline (TBS, pH = 7.4), the test and control slides were incubated for 15 minutes with Powervision^{+TM} Post-antibody blocking solution (Immunovision Technologies, Daly City, CA, USA). Next, slides were washed and incubated with Powervision^{+TM} polymerized horseradish peroxidase conjugates (Immunovision Technologies, Daly City, CA, USA). Finally, the sections were stained with 3, 3'-diaminobenzidine tetrahydrochloride (Sigma, Zwijndrecht, NL) as chromogen, counterstained with Mayer's hematoxylin and visualized with light microscopy.

Quantitative scoring analysis of immunohistochemistry

Prior to screening, sections were coded so that the observers were unaware of the clinical details of the case under study. Expression of VEGF, Flt-1 and KDR/Flk-1 was analyzed semi-quantitatively, using a visual scoring method with grades ranging from 0 to 3 (0 = no staining; 1 = moderate staining; 2 = intense staining; 3 = very intense staining) as previously described (8, 19, 20, 24). The entire section of a tissue block was investigated and scored at the same magnification. The staining intensity of VEGF, Flt-1 and KDR/Flk-1 was scored blindly by two independent observers, who were unaware of the clinical data of the case under study, in bronchial and bronchiolar airways as well as alveolar parenchyma in cells of epithelial, endothelial and smooth muscle origin as well as macrophages. We examined errors within and between observers by correlating the expression scores using Pearson's analysis and found a very high correlation ranging from 0.8 to 0.9. In the bronchial airways staining was assessed in the bronchial epithelium, mucosal microvasculature, submucosal bronchial wall vessels, airway smooth muscle (ASM) cells and macrophages in the bronchial airway wall. In peripheral lung tissues the staining of VEGF and receptors was analyzed in bronchiolar an alveolar epithelium, bronchiolar ASM cells, and bronchiolar and alveolar macrophages. The vasculature in the peripheral lung was further subdivided into the larger pulmonary vessels associated with the bronchiolar airways and smaller vessels situated within the alveolar parenchyma. In each the VEGF and receptor staining of endothelial, intimal and medial VSM cells were assessed.

Since TGF-β_1 may also induce VEGF expression in epithelial cells (25, 26), we assessed the correlation between the epithelial VEGF expression from the current study

and epithelial TGF-β_1 expression from one of our previous studies (20). In both studies the same patient groups were used and the staining was performed on adjacent or near sections.

Statistical Analysis

Data were analyzed for statistical significance using the unpaired, two-tailed Students' t-test as well as the non-parametric Mann-Whitney test, where appropriate. The expression data for VEGF and its receptors were expressed as mean ± SEM. Furthermore, VEGF and its receptors staining for different compartments were correlated with FEV_1 using Pearson's correlation analysis. Differences with $p \leq 0.05$ were considered to be statistically significant.

3.4 Results

Clinical Parameters

The clinical and lung function characteristics of all subjects included in the study are listed in Table 3.1. As defined, the COPD group demonstrated decreased FEV_1 and FEV_1/FVC values, ($p<0.001$) as has been described previously (19-21). The subjects in the two groups did not differ significantly in age and smoking status (pack-years) or steroid use (Table 3.1).

TABLE 3.1 A summary of the clinical characteristics of subjects with and without chronic obstructive pulmonary disease

Group	Sex (M/F)	Age	PY	FEV_1 (% Pred.)	FEV_1/FVC (%)	Steroid treatment
Non-COPD	10/4	64 (3.7)	42 (7.7)	101 (3.3)	0.72 (0.02)	None
COPD	14/0	64 (2.3)	44 (0.8)	63 (2)	0.54 (0.02)	4
p-value		0.84	0.82	< 0.001	< 0.001	

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; Forced expiratory volume in 1 second (FEV_1) and Forced vital capacity (FVC) are given as percentages of the predicted values (% Pred.) before bronchodilatation. M = Male; F = Female. PY = number of pack years. Data shown represent means with standard deviation in brackets. The patients in these two groups participated in a larger project, part of which has been published previously (19-21).

Immunolocalization of VEGF, Flt-1 and KDR/Flk-1

Bronchial airways

Examples of VEGF expression in central airways of non-COPD and COPD subjects are given in Figure 3.1A and 3.1B, whereas 3.1C and 3.1D (both taken from COPD subjects) show the VEGF receptors Flt-1 and KDR/Flk-1, respectively. In all subjects, within the airways VEGF, Flt-1 and KDR/Flk-1 were localized in the bronchial epithelium and airway smooth muscle (ASM) cells, bronchial microvasculature of mucosa and submucosa and on inflammatory cells, predominantly macrophages, (Figure 3.1A-D). In the vessel wall, vascular smooth muscle (VSM) cells were positive for VEGF, Flt-1 and KDR/Flk-1, whereas endothelial cells did not stain for VEGF protein but were positive for the Flt-1 and KDR/Flk-1 (Figure 3.1). To assess the intensities of VEGF, Flt-1 and KDR/Flk-1 expression in various bronchial airway compartments, we opted for a visual scoring method as previously described (8, 19, 20, 24). VEGF expression was increased in bronchial airway smooth muscle cells of COPD patients as compared to non-COPD subjects (1.6 fold, $p<0.01$) but not in bronchial epithelial cells and macrophages (Figure 3.2A). In the central airways of patients with COPD as compared to non-COPD subjects, VEGF staining was more intense in VSM of microvasculature the bronchial mucosal (lamina propria) (1.7 fold, $p<0.001$) and bronchial VSM in the submucosa (1.4 fold, $p<0.01$, Figure 3.2A). No significant differences were observed when considering the expression levels of KDR/Flk-1 and Flt-1 between COPD subjects and non-COPD patients (Figure 3.2B and 2C, respectively). In all subjects VEGFR-2 (KDR/Flk-1) expression was more intense than VEGFR-1 (Flt-1) expression, except for the expression in endothelial cells of bronchial microvessels and on bronchial macrophages, which were comparable (Figure 3.2B and 3.2C).

Bronchiolar airways

Figure 3.3 shows photographs of peripheral lung tissues from non-COPD and COPD subjects for VEGF (3.3A and 3.3B), KDR/Flk-1 (3.3C and 3.3D) and Flt-1 (3.3E and 3.3F), respectively. In bronchiolar epithelial cells VEGF (1.5 fold, $p<0.001$, Figure 3.4A) and Flt-1 expression (1.4 fold, $p<0.04$, Figure 3.4C) were increased in COPD patients as compared to non-COPD subjects, whereas the staining for KDR/Flk-1 was unchanged between both patient groups (Figure 3.4B).

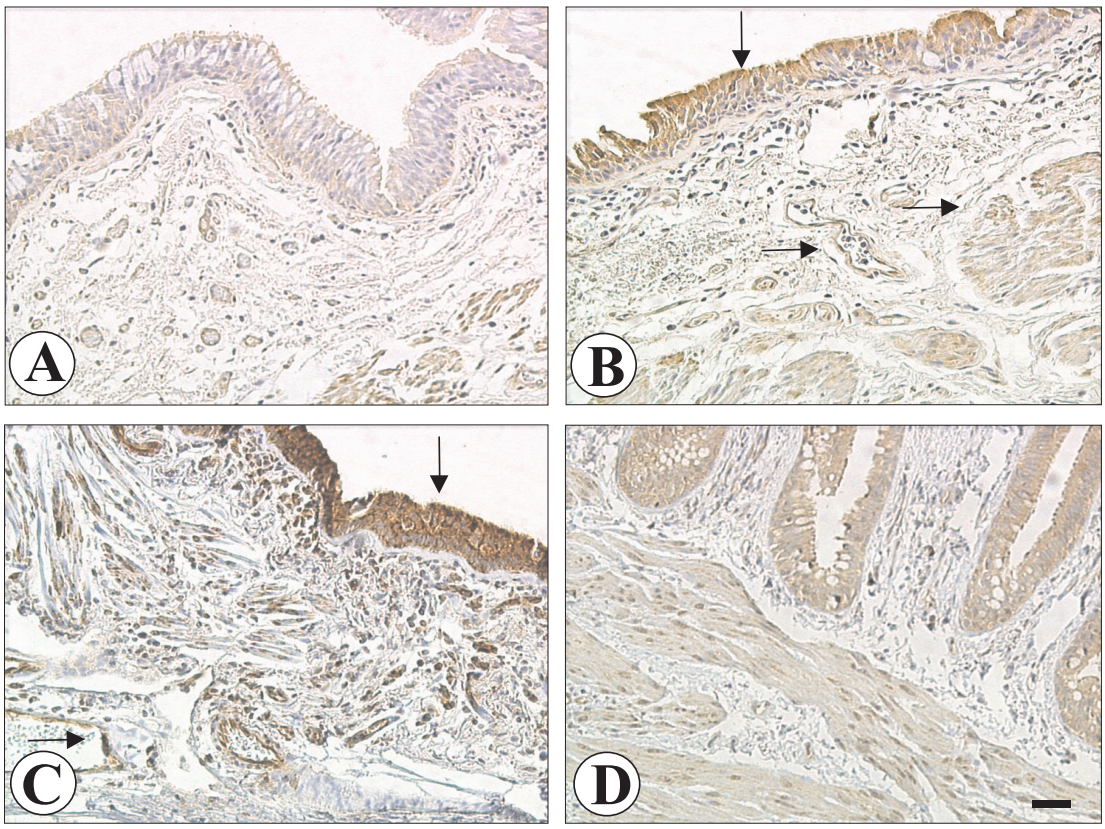

Figure 3.1 Immunohistochemical localization of VEGF (**A-B**), KDR/flk-1 (**C**) and flt-1 (**D**) in bronchial tissues from non-COPD (ex-) smoking subjects (A) and patients with COPD (B, C, D). Immunoreactive VEGF, KDR/flk-1 and flt-1 were localized in bronchial epithelial cells, airway smooth muscle (ASM) cells and in macrophages, endothelial and vascular smooth muscle (VSM) cells. Color is developed with 3, 3-diaminobenzidine tetrahydrochloride (DAB) as chromogen (brown color) and counterstained with Mayer's hematoxylin. Arrows indicate sites of positivity for VEGF, flt-1 or KDR/flk-1. Original magnification: x100; Scale bar = 50 μm.

Airway smooth muscle cells showed slightly increased VEGF expression in bronchiolar region (1.3 fold, $p<0.05$), whereas the expression of both the receptors remained unchanged in two patient groups. However, the expression of KDR/Flk-1 was more intense than Flt-1 in all patients (Figure 3.4B and 3.4C).

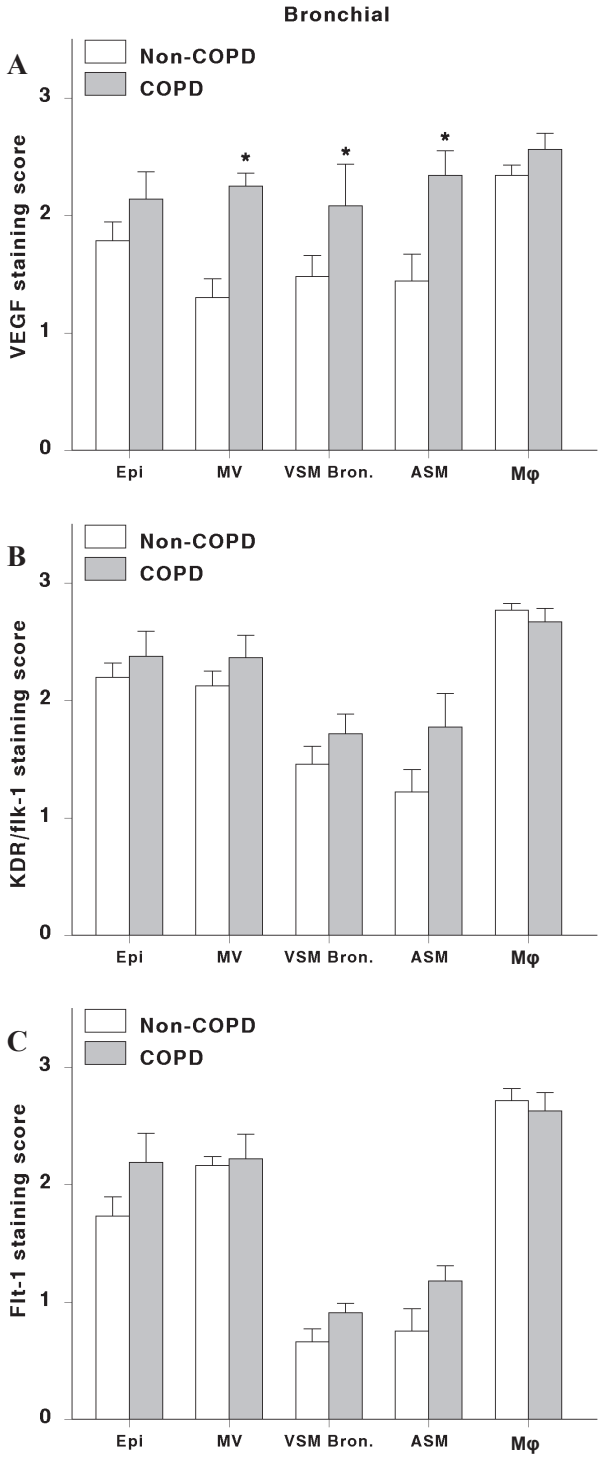

Figure 3.2 Graphic representations of VEGF (**panel A**), KDR/flk-1 (**panel B**) and flt-1 (**panel C**) protein expression in different cell types in bronchial airways using visual scoring. The immunostaining score ranges from 0 (no staining) to 3 (very intense staining). Open and closed bars represent mean data from subjects without and with COPD, respectively. Data are presented as mean ± S.E.M. An asterisk indicates a significant difference ($p<0.05$, Student's t-test) as compared to non-COPD subjects. Abbreviations: bronchial epithelium (Epi), bronchial microvessels (MV) in the mucosa, bronchial vascular smooth muscle cells (VSM) in the submucosa, airway smooth muscle (ASM) and macrophages (Mφ).

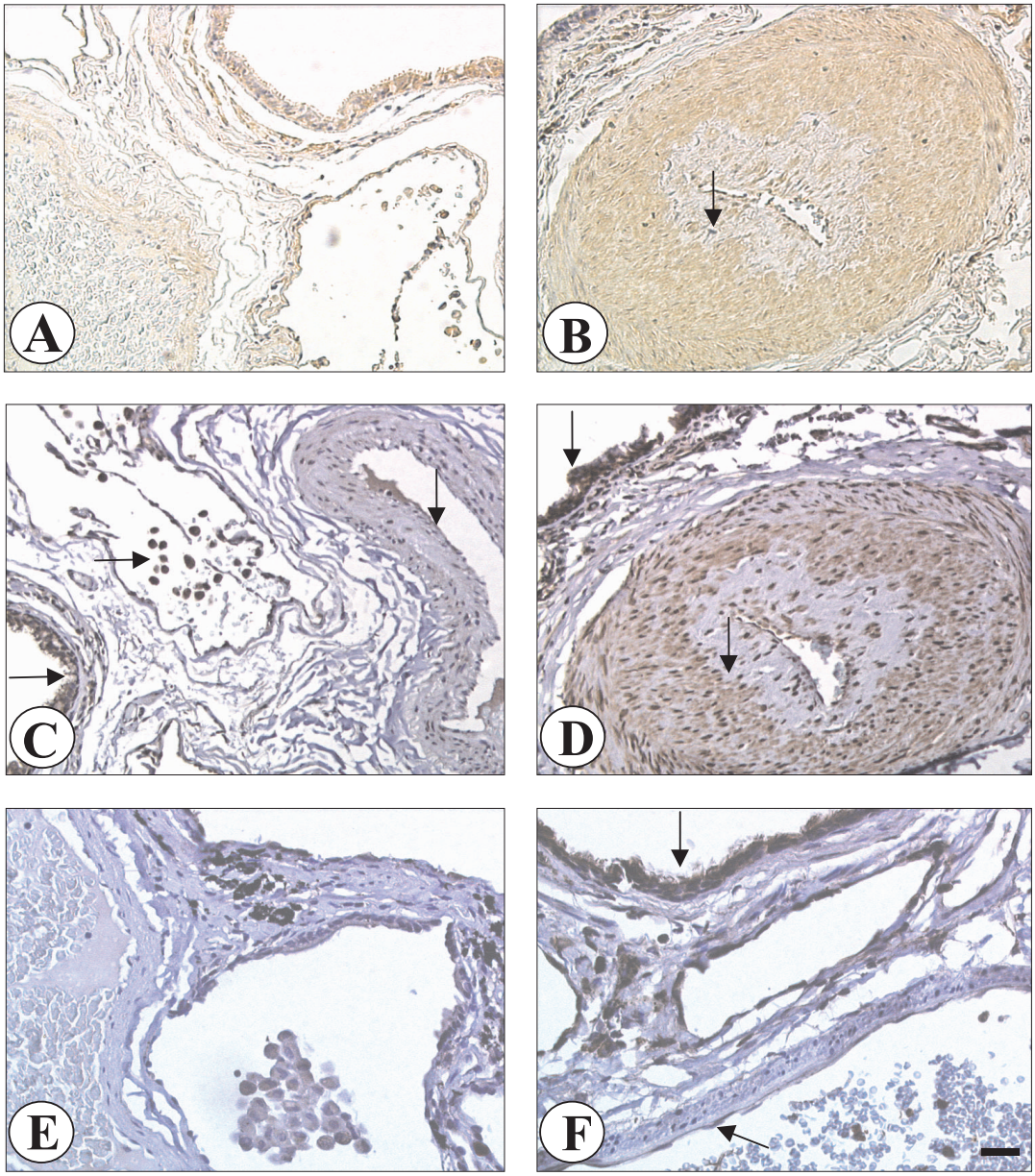

Figure 3.3 Immunohistochemical localization of VEGF (**A-B**), KDR/flk-1 (**C-D**) and flt-1 (**E-F**) in peripheral tissues from non-COPD (ex-) smoking subjects (A, C, E) and patients with COPD (B, D, F). Immunoreactive VEGF, flt-1 and KDR/flk-1 were localized in bronchiolar and alveolar epithelial cells, airway smooth muscle (ASM) cells, macrophages and in endothelial and intimal/medial vascular smooth muscle (VSM) cells. Color is developed with 3, 3-diaminobenzidine tetrahydrochloride (DAB) as chromogen (brown color) and counterstained with Mayer's hematoxylin. Arrows indicate sites of positivity for VEGF, flt-1 or KDR/flk-1. Original magnification: x100; Scale bar = 50 μm.

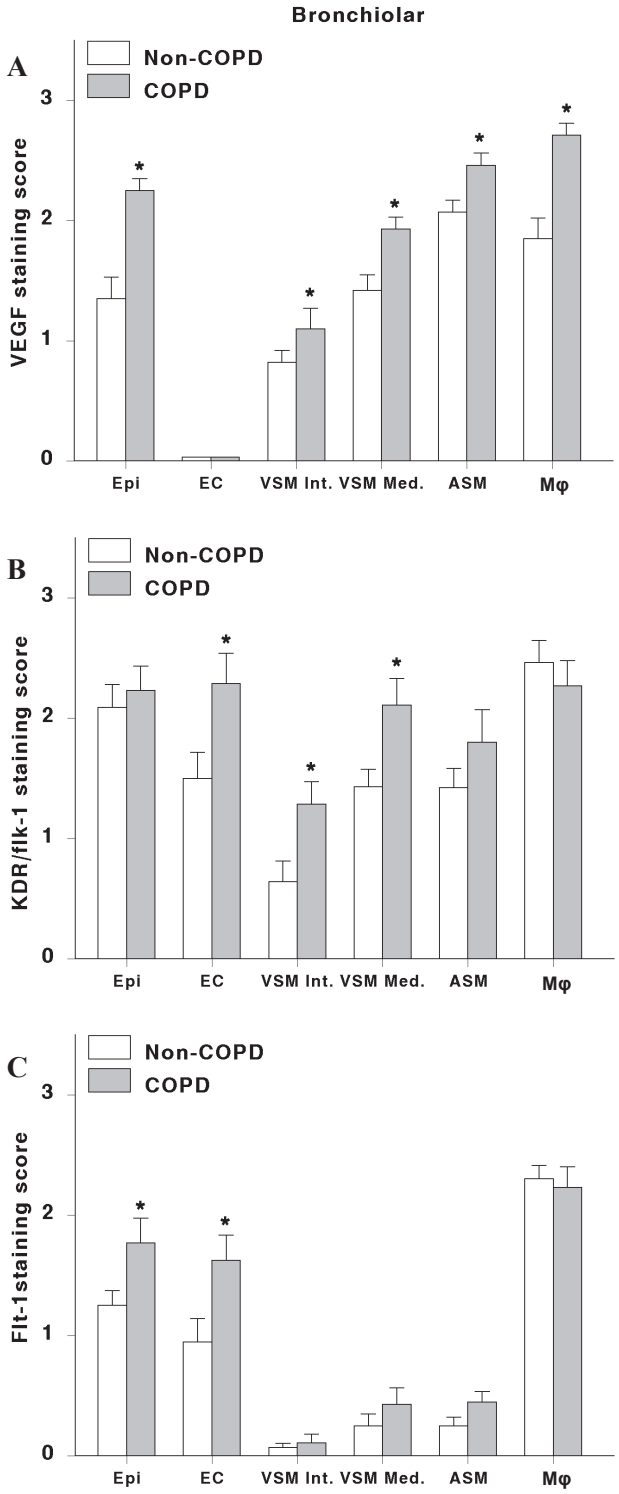

Figure 3.4 Graphic representations of VEGF (**panel A**), KDR/flk-1 (**panel B**) and flt-1 (**panel C**) protein expression in different cell types in bronchiolar airways and associated pulmonary arteries using visual scoring. Open and closed bars represent mean data from subjects without and with COPD, respectively. Data are presented as mean ± S.E.M. An asterisk indicates a significant difference ($p<0.05$, Student's t-test) as compared to non-COPD subjects. Abbreviations: bronchiolar epithelium (Epi), endothelial cells (EC), intimal and medial vascular smooth muscle cells (VSM int. and med.), airway smooth muscle (ASM) and bronchiolar macrophages (Mφ).

When considering the expression of VEGF in the larger pulmonary arteries associated with the bronchiolar airways, the fold in intimal and medial VSM staining was 1.7 and 1.3 ($p<0.05$, Figure 3.4A) between COPD and control subjects respectively, whereas endothelial cells did not express VEGF. KDR/Flk-1 expression was enhanced in endothelial cells, intimal and medial VSM (1.3, 1.9 and 1.5 fold, $p<0.02$, Figure 3.4B), whereas the corresponding value in endothelial cells for Flt-1 expression is 1.7 ($p<0.03$, Figure 3.4C). In both patient groups, the intimal VSM stained 2-3 times less intense than medial VSM for VEGF, Flt-1 and KDR/Flk-1. Moreover, the vascular Flt-1 expression was lower than KDR/Flk-1 and VEGF in each of the investigated vessel wall areas ($p<0.002$, Figure 3.4). Staining of VEGF in bronchiolar macrophages (1.5 fold, $p<0.001$, Figure 3.4A) was increased in COPD as compared to non-COPD subjects, whereas the staining on macrophages of Flt-1 or KDR/Flk-1 expression in bronchiolar airways as well as VEGF, Flt-1 or KDR/Flk-1 in the alveolar region remained unchanged (Figure 3.5).

Alveolar parenchyma

Staining of alveolar epithelial cells (type I and II) for COPD was more intense than for non-COPD controls (1.5 fold, $p<0.0001$, Figure 3.5A). KDR/Flk-1 and Flt-1 expression were not changed in alveolar epithelial cells (Figure 3.5B and 3.5C). VEGF expression was increased in intimal and medial VSM (1.5 and 1.7 fold, $p<0.01$, Figure 3.5A) of small pulmonary vessels in the alveolar region whereas the corresponding values for KDR/Flk-1 were 2.0 and 1.8 ($p<0.02$), respectively (Figure 3.5B). Furthermore, the expression of both KDR/Flk-1 and Flt-1 were increased in endothelial cells of small pulmonary vessels in lung parenchyma (1.7 and 2.1 fold, $p<0.001$, Figure 3.5B and 3.5C).

Correlation between staining and clinical data

We examined the relation between FEV_1 values of patients in both groups and the staining scores of VEGF, Flt-1 and KDR/Flk-1 in the investigated areas. Within the bronchial airways, FEV_1 values were inversely correlated with VEGF staining sores in bronchial mucosal microvasculature ($r = -0.65$; $p<0.001$, Figure 3.6A), bronchial ASM cells ($r = -0.45$; $p<0.01$, Figure 3.6B) if all subjects were analyzed together.

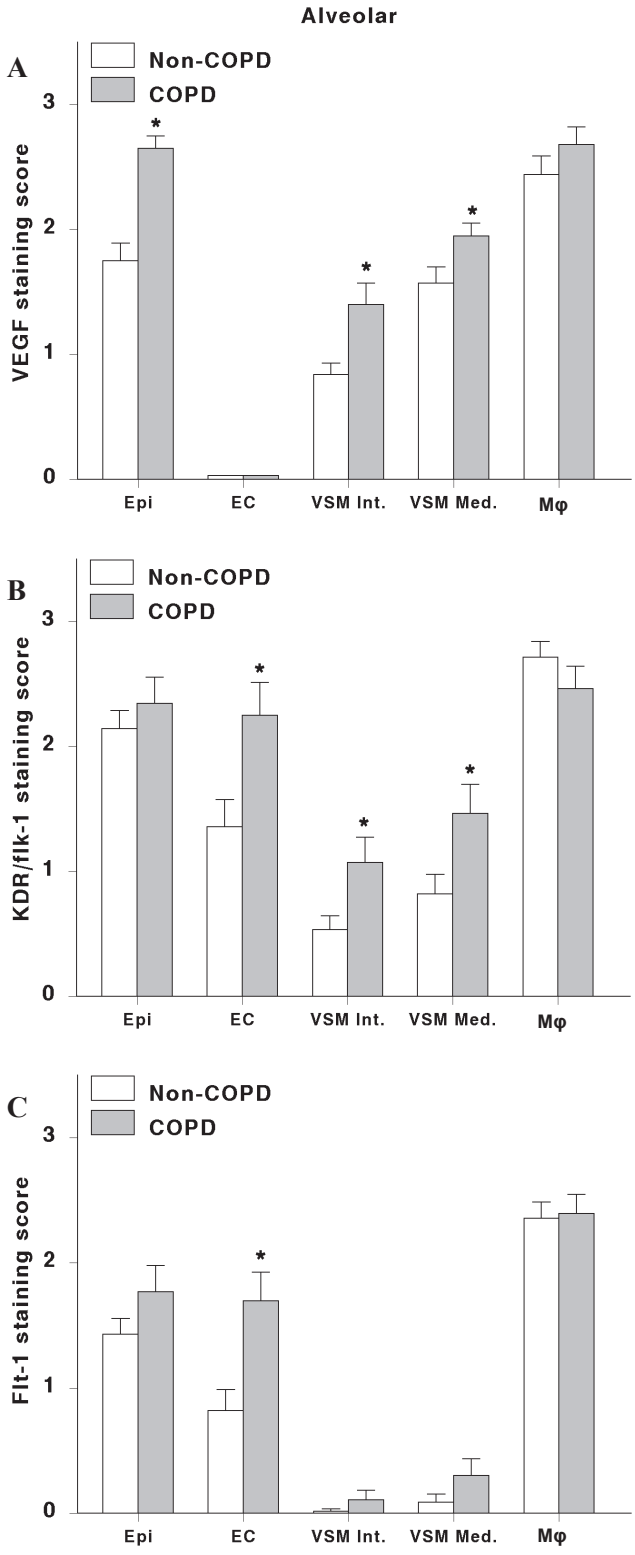

Figure 3.5 Graphic representations of VEGF (**panel A**), KDR/flk-1 (**panel B**) and flt-1 (**panel C**) protein expression in different cell types in alveolar parenchyma and pulmonary vasculature using visual scoring. Open and closed bars represent mean data from subjects without and with COPD, respectively. Data are presented as mean ± S.E.M. An asterisk indicates a significant difference ($p<0.05$, Student's t-test) as compared to non-COPD subjects. Abbreviations: bronchiolar epithelium (Epi), endothelial cells (EC), intimal and medial vascular smooth muscle cells (VSM int. and med.), and alveolar macrophages (Mφ).

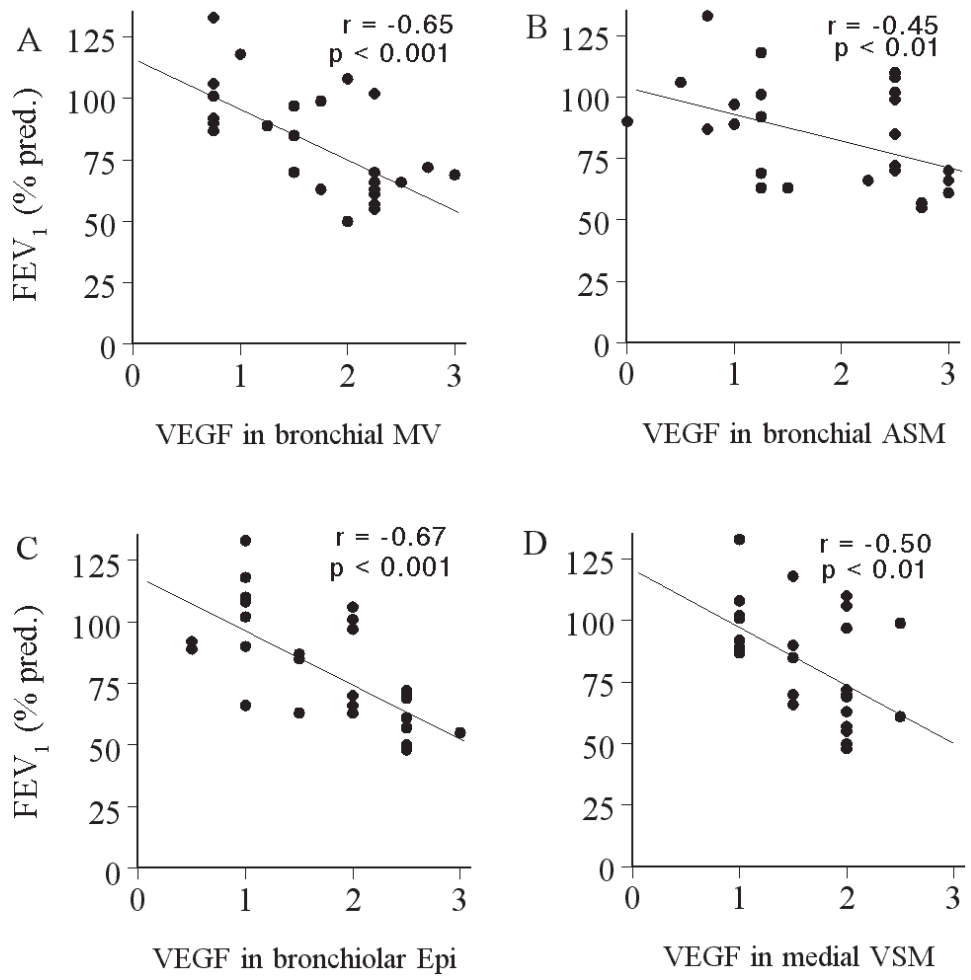

Figure 3.6 **Correlation with FEV_1 (% predicted) of VEGF protein expression** in microvessels (MV) in the bronchial mucosa (***A***), bronchial airway smooth muscle (ASM) cells (***B***), bronchiolar epithelial (Epi) cells (***C***) and medial vascular smooth muscle (VSM) cells of pulmonary arteries associated with the bronchiolar airways (***D***). Correlation was assessed for the combined patient groups (non-COPD and COPD). Correlation coefficient (r) was obtained using linear regression (Pearson's) analysis.

The bronchiolar epithelium ($r = -0.67$; $p<0.001$, Figure 3.6C) and medial VSM of larger pulmonary arteries associated with bronchiolar airways ($r = -0.50$; $p<0.01$, Figure 3.6D) also showed an inverse correlation with FEV_1 values from the total group. Additionally, VEGF expression in medial VSM was correlated with KDR/Flk-1 expression in endothelium of pulmonary arteries ($r = 0.41$; $p<0.01$) as well as smaller alveolar vessels ($r = 0.48$; $p<0.01$). Furthermore, we found correlation for the expression pattern of KDR/Flk-1 and Flt-1 in the endothelium of pulmonary arteries ($r = 0.67$; $p<0.001$) as well as in alveolar vessels ($r = 0.80$; $p<0.0005$).

Additionally, we examined correlation between the epithelial VEGF expression from the current study and epithelial TGF-β_1 expression from one of our previous studies (20). In both studies the same patient groups were used and the staining was performed on adjacent or near sections. With regard to the bronchiolar epithelium, Pearson's analysis revealed a significant positive correlation between the VEGF protein and TGF-β_1 protein levels ($r = 0.55$; $p<0.004$) and VEGF protein and TGF-β_1 mRNA expression ($r = 0.45$; $p<0.02$). With regard to the alveolar epithelium, the VEGF protein levels correlated significantly with the TGF-β_1 mRNA expression only ($r = 0.58$; $p<0.002$), but not with the TGF-β_1 protein levels ($r = 0.31$; $p<0.12$).

3.5 Discussion

In this study we show that COPD is associated with an increased expression of VEGF in the bronchial, bronchiolar and alveolar epithelium and in bronchiolar macrophages as well as ASM and VSM cells in both bronchiolar and alveolar region. KDR/Flk-1 and Flt-1 were increased in COPD as compared to non-COPD in endothelial, intimal and medial VSM cells of larger pulmonary arteries and of smaller caliber alveolar vessels. Interestingly, we observed a significant inverse correlation of VEGF with FEV_1 in bronchial mucosal microvessels and ASM cells, bronchiolar epithelium and medial VSM of larger pulmonary arteries associated with bronchiolar airways. TGF-β_1 staining in the bronchiolar epithelium also correlated with VEGF in the same patients as described in our previous study (20).

Our results indicate that VEGF and its receptors Flt-1 and KDR/Flk-1 are localized within the airways and vasculature in endothelial and epithelial cells as well as smooth muscle cell origin and furthermore on various inflammatory cells, predominantly macrophages. The localization of VEGF and its receptors in the lungs of our patient groups is in agreement with earlier reports, which described a similar staining pattern in human developing and normal adult as well as in emphysematous lungs (17, 27, 28). In contrast to Kasahara et al. (28), where authors showed in emphysematous lungs that VEGF and its receptor VEGF-R2 were decreased in total lung extracts, as measured with ELISA or western blot analysis, we found that the epithelial and endothelial cells in the alveolar spaces and in the most distal airways were intensely positive for VEGF and KDR/Flk-1 in COPD patients. Furthermore, our patient groups could be considered as mild to moderate COPD whereas, in the study of

Kasahara the selected patients were solely emphysematous in origin. Our findings of increased VEGF expression in viable cell populations represent in part a successful attempt to repair sustained damage and perhaps contribution to vascular remodeling and their participation in the establishment and maintenance of the functional blood-gas interface, maturation, survival and proliferation of capillary endothelial cells (29). In adult lungs, VEGF and its receptor system could contribute in the maintenance of endothelial and epithelial cell viability in response to injury (31).

Interestingly, immunoreactivity for VEGF in intimal and medial VSM cells and for Flt-1 as well as KDR/Flk-1 in endothelial cells of pulmonary arteries and alveolar vessels was elevated in patients with COPD. The highest levels of VEGF expression in the pulmonary vasculature were observed in the medial VSM cells and of KDR/Flk-1 in endothelial cells of arteries with a diameter of approximately 200 μm which are known to play an important role in pulmonary blood pressure regulation and vascular resistance (14, 30). Pulmonary hypoxia and hypertension with increased sheer stress are pathophysiological conditions that have been shown to increase the expression of VEGF in VSM cells (13, 14). Blockade of KDR/Flk-1 is associated with obliterative endothelial cell proliferation in pre-capillary arterioles with abnormal vessel development and at the same time with induction of capillary endothelial and cell death by apoptosis, together leading to death in rat embryos, similar to that seen in human primary pulmonary hypertension subjects (13, 18, 31, 32). In a follow-up study they found that after VEGFR-2 blockade apoptosis predominated in areas of oxidative stress and that apoptosis blockade by a broad spectrum caspase inhibitor markedly reduced the expression of markers of oxidative stress (33). Hypoxia, oxidative stress and pulmonary hypertension are pathological features often seen in advanced COPD patients and increased VEGF expression may lead to increased or even abnormal proliferation of endothelial and VSM cells in pulmonary vessels. This suggests a potential role of this endothelial mitogen in peripheral angiogenesis and vascular remodeling, possibly in orchestration with other smooth muscle specific growth factors like FGF-2, PDGF and TGF-$ß_1$ (12, 34-36).

We observed increased expression for VEGF and unchanged expression levels for Flt-1 and KDR/Flk-1 in bronchiolar and alveolar epithelial cells as well as in airway smooth muscle cells in COPD. It has been previously documented that the expression of VEGF and receptor KDR/Flk-1 can also be induced by stimuli like

hypoxia and oxidative stress in other than endothelial cells, such as epithelial and smooth muscle cells (33, 37, 38). In a recent report, Kanazawa and colleagues (39) have demonstrated that VEGF levels in induced sputum were higher in patients with bronchitis and lower in emphysema as compared to normal controls. Moreover, VEGF levels in bronchitis patients were inversely correlated with FEV_1 values. Our data on inverse correlation of VEGF levels in various airway and vascular cells is in agreement with this report. In our study subjects with COPD could not be subdivided into patients with either chronic bronchitis or emphysema alone. Furthermore, the nature of the human material examined (sputum) in the study of Kanazawa and colleagues is different than the lung tissue where we immunohistochemically localize and quantify the VEGF and its receptor levels.

Recent studies indicated that the expression of VEGF was increased in bronchial and alveolar epithelial cells and also was induced in α-SMA positive (myo-)fibroblasts in bleomycin induced fibrosis in the rat and in human patients with pulmonary fibrosis and that these fibrotic regions were densely populated by mast cells and macrophages with elevated KDR/Flk-1 expression (15, 17). We have shown earlier that mast cells and macrophages were increased in bronchiolar airway epithelium and reported an increased expression of TGF-$ß_1$ in bronchiolar and alveolar epithelial cells in patients with COPD (20, 21). We found a significant correlation between VEGF expression in epithelial cells with the expression of TGF-$ß_1$ published on same patient groups earlier (20) suggesting that the VEGF/Flk-1 system, possibly together with TGF-$ß_1$, represents a molecular link between inflammatory cell accumulation and proliferation of myo-fibroblasts. Summarizing, the elevated VEGF and TGF-$ß_1$ expression on bronchiolar epithelial cells and macrophages and the presence of KDR/Flk-1 and Flt-1 suggests a mechanism of initiating and perpetuating fibrosis at sites of tobacco induced injury contributing to airway remodeling in COPD. As inhaled corticosteroids could decrease the VEGF expression levels (40), but this was not the case in our study as none of the patient received inhaled corticosteroid therapy except 4 patients received corticosteroids perioperatively. However, caution must be exercised in extrapolating the expression data based on fourteen patients in each group as the increased trend of VEGF expression in bronchial airways and KDR/Flk-1 in bronchial and bronchiolar airway smooth muscle could reach significance if more patients would have been examined.

Taken together, these findings strongly suggest a role for VEGF and its receptors in airway and vascular remodeling, and thereby in the development of airway obstruction in COPD. At present, our knowledge of airway and vascular remodeling during the development of COPD is far from complete. Probably, many growth factors, among them VEGF, play an essential role in the pulmonary and vascular viability and repair in response to tissue injury. The increased pulmonary VEGF expression in airways, parenchymal lining and small-diameter pulmonary vessels in COPD may reflect an, in part unsuccessful, attempt to stimulate tissue repair mechanisms caused by tobacco-induced injury.

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Chapter 4

FGF-FGFR$_1$ system and Airway Remodeling in COPD

Adapted from:

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4.1 Summary

An important feature of chronic obstructive pulmonary disease (COPD) is airway remodeling of which the molecular mechanisms are poorly understood. We assessed the role of fibroblast growth factors (FGF-1 and FGF-2) and receptor, FGFR-1 in bronchial airway wall remodeling in patients with COPD (FEV_1 <75%; n=15) and without COPD (FEV_1 >85%; n=16). FGF-1 and FGFR-1 were immunolocalized in bronchial epithelium, airway smooth muscle (ASM), submucosal glandular epithelium and vascular smooth muscle. Quantitative digital image analysis revealed increased cytoplasmic expression of FGF-2 in bronchial epithelium (0.35±0.03 vs. 0.20±0.04, $p<0.008$) and nuclear localization in ASM ($p<0.0001$) in COPD patients as compared to controls. Elevated levels of FGFR-1 in ASM ($p<0.005$) and of FGF-1 ($p<0.04$) and FGFR-1 ($p<0.001$) in bronchial epithelium were observed. In cultured human ASM cells, FGF-1 and/or FGF-2 (10 ng/ml) induced cellular proliferation, as shown by ^{3}H-thymidine incorporation assay and by cell number counts. Steady state mRNA levels of FGFR-1 were elevated in human ASM cells treated with either FGF-1 or FGF-2. The increased bronchial expression of fibroblast growth factors and their receptor in patients with COPD, and the mitogenic response of human ASM cells to FGFs *in vitro,* suggest a potential role for FGF/FGFR-1 system in the remodeling of bronchial airways in COPD.

4.2 Introduction

Chronic obstructive pulmonary disease (COPD) is a global health problem with increasing morbidity and mortality (1). One of the major determining factors is tobacco smoking (2). However, only ten percent of all smokers develop COPD. One of the key pathological features of COPD is thickening of airway walls, which is thought to be a result of a chronic smouldering inflammatory process, in which neutrophils, macrophages and T-lymphocytes play a role, and which is associated with hyperplasia of airway smooth muscle cells and (myo-)fibroblasts, and increased deposition of extracellular matrix (3). The bronchial epithelium and airway smooth muscle are two major cellular structures involved in airway remodeling (3). A variety of growth factors and cytokines including platelet–derived growth factor-B (PDGF-B) and epidermal growth factor (EGF), transforming growth factor-β (TGF-β) that are

released from these sites of the airway wall have the potential to contribute to the pathogenesis of airway remodeling (4-6). Supporting *in vitro* evidence for a relationship between epithelial injury and enhanced airway remodeling is provided by studies of co-cultures from bronchial epithelial cells and myo-fibroblasts (7, 8): these studies revealed enhanced cellular proliferation and increased collagen expression resulting from the interaction of these cells with several growth factors, including basic FGF (FGF-2), insulin-like growth factor-1, PDGF-B, TGF-β, endothelin-1 and EGF.

Fibroblast growth factors (FGFs) may well play a pivotal role in regulating the airway wall remodeling. A number of studies have demonstrated that members of EGF and FGF family contribute to chronic inflammatory and tissue repair processes as well as to fibrosis in chronic airway diseases such asthma (9, 10). Fibroblast growth factors bind to four high-affinity, transmembrane tyrosine-kinase receptors (FGFR1-4). Distinct FGF subtypes bind with different affinity to the various FGF receptors. Alternative splicing and regulated protein trafficking further modulate the intra-cellular events initiated by FGF ligand-receptor interaction (11). Increased expression of FGF-1 and FGFR-1 has been shown during the development of lung fibrosis (12) and FGF-2 has been implicated in the pathogenesis of obliterative bronchiolitis in lung transplants (13).

We postulate that the FGF-FGFR system is involved in the pathogenesis of COPD. We investigated the expression patterns of FGF-1, FGF-2 and FGFR-1 in bronchial airways of (ex-) smokers with or without COPD. In addition, we examined the cell proliferation and the expression of FGFR-1 in cultured human ASM cells stimulated with FGF-1 and FGF-2.

4.3 Materials and methods

Selection of Specimens

The Medical Ethics Committees of the Leiden University Medical Center and Southern Hospital Rotterdam, The Netherlands approved the study. Lung tissue from the hospitals pathology archives was obtained from patients who underwent lobectomy or pneumonectomy. Based on lung function data, patients were assigned (6, 14) to the *COPD group* (n = 15) consisting of fifteen subjects with forced one-

second expiratory volume (FEV_1) <75% of predicted value (15) before bronchodilatation, FEV_1/FVC ratio <75%, a reversibility in FEV_1 ≤12% of predicted after 400 μg inhaled salbutamol, and with a carbon monoxide diffusion capacity (K_{co}) ≤80% of predicted value or to the *Non-COPD group* (n = 16) consisting of sixteen subjects with FEV_1 >85% before bronchodilatation, FEV_1/FVC ratio >85% and the total lung capacity (TLC) of over 80% (15). The patients in these two groups participated in a larger research project, part of which has been published previously (16, 17). Clinical data of all patients were examined for possible co-morbidity and medication usage. All pulmonary function tests were performed within 3 months prior to surgery as described earlier (16). Lung function data and other patient characteristics are shown in Table 4.1.

Immunohistochemistry

Serial sections of 4 μm were deparaffinized, rehydrated and immunostained using a Multilink® labelling system (Biogenex, San Ramon, USA) and specific anti-human mouse monoclonal antibodies against α-smooth muscle actin (α-SMA, NeoMarkers, Fremont, USA), Ki-67 (Biogenex, San Ramon, USA), FGF-2 (Transduction Laboratories, Lexington, USA), FGF-1 and FGFR-1 (kind gift from Dr. J. Walters) as described previously (18, 19). Color was developed using New Fuchsin or 3, 3-diaminobenzidine as chromogens. Slides were counter stained with Mayer's hematoxylin. Positive controls consisted of human breast carcinoma and placental tissue. The optimal dilutions for all antibodies were identified by examining the intensity of staining obtained with a series of dilutions: the optimum concentration resulted in specific and easily visible signal on control specimens. Negative controls consisted of omission of the primary antibody.

Quantitative analyses of immunostaining

Digital images (pixel size: 736x574) from each subject were analysed using Leica Qwin image analysis system (Leica BV, Rijswijk, The Netherlands). Staining patterns of FGF-1, FGF-2, FGFR-1 and α-SMA were analysed by interactively drawing areas and assessing the area of positive staining divided by the total measured cellular area of the respective epithelial or ASM layer. The nuclear localization of FGF-2 in ASM was assessed by computerized counting of individual nuclei and the data is expressed

as the number of positive nuclei divided by total nuclei (labelling index, LI). In case of vascular expression of FGFs and FGFR-1, quantitative analysis was performed using an arbitrary visual scale with grading scores of 0, 1, 2, and 3 (Figure 4.1) representing none (panel A), weak (panel B), moderate (panel C) and intense (panel D) staining, respectively (6, 14).

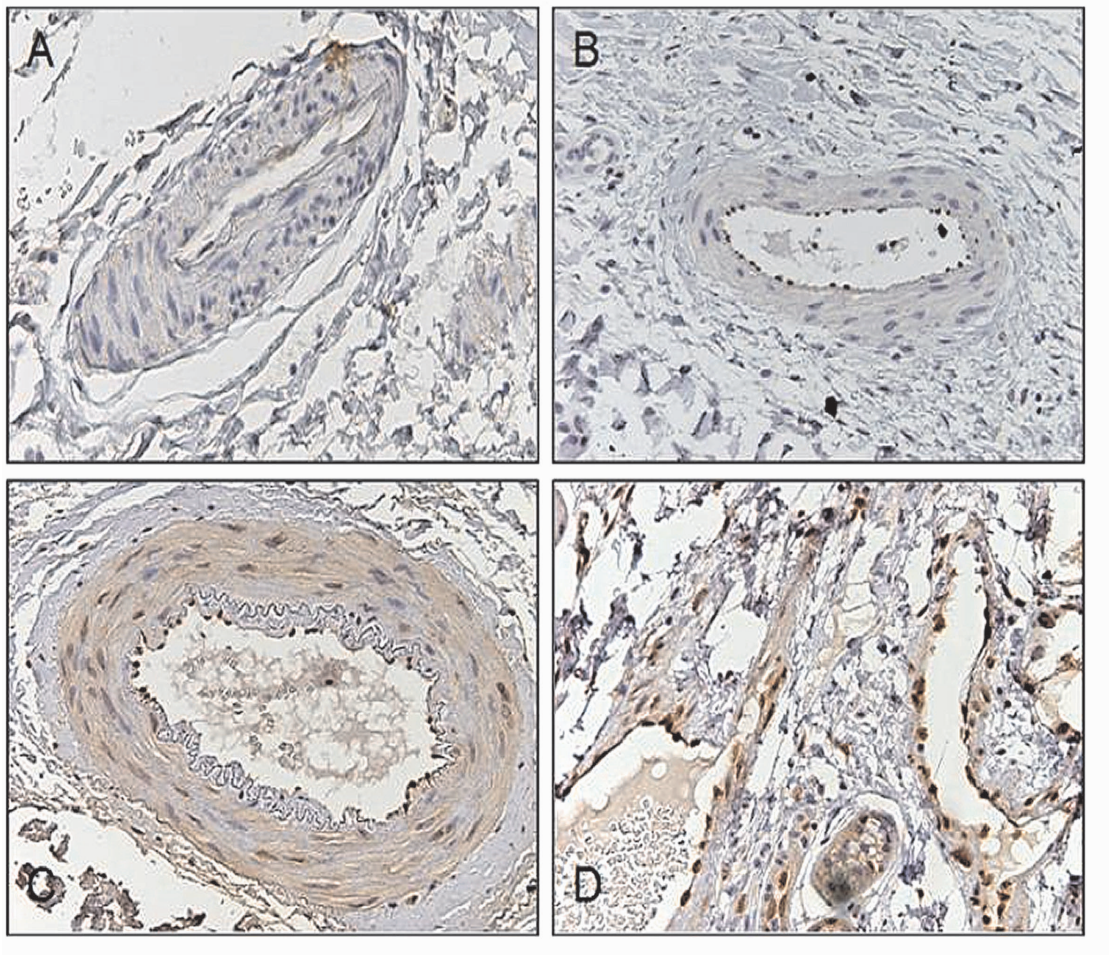

Figure 4.1 **Immunohistochemical localization of FGF-2 in bronchial vessels.** Representative examples of staining intensity pattern used for visual scoring. Photomicrographs depict lung tissue sections from patients without COPD (***A*** and ***B***) and with COPD (***C*** and ***D***) showing nuclear staining of FGF-2 in vascular smooth muscle cells. Panels ***A*** to ***D*** show representative examples of staining intensities used for visual scoring, 0-3 respectively. Original magnification: x100.

Isolation and culture of human ASM cells

Human airway smooth muscle cells were from three different non-asthmatic, non-COPD and (ex) smoker donors who underwent lobectomy or pneumonectomy as described previously (20, 21). ASM cells were immunocytochemically characterised (α-SMA and smooth muscle myosin heavy chain staining) and used for experiments at passage 4-5.

ASM cell Proliferation assays

Cells were seeded at a density $1x10^4$ cells/well in 96-wells plates, cultured until confluence, subsequently serum deprived to synchronise the growth and incubated with either 0.1, 1.0, 10, or 50 ng/ml human recombinant FGF-1 (Promega, Madison, USA) and/or FGF-2 (Sigma-Aldrich, St. Louis, USA) for 8, 24 and 48 h. Control cells received FBS-free DMEM alone. Five hours prior to the end of the treatment, 1μCi/well of [^{3}H]-thymidine (Amersham, Roosendaal, the Netherlands) was added. The cells were harvested on glass fiber filters and radioactivity was assessed using a Microplate Scintillation β-counter (Topcount, Packard, Meridan, USA). The mean CPM of quadruple wells and subsequently from three different cell batches was expressed as fold change compared to controls. In a parallel series of experiments, cells in quadruple were stimulated for 24 and 48 hours and processed for cell counting in the Casey®1 system (Schärfe system GmbH, Reutingen, Germany) (20).

RNA isolation and RT-PCR

Growth–arrested ASM cells were incubated with either FGF-1 or FGF-2 (10 ng/ml) for 1, 2, 4, 8, 24 and 48 h. Total RNA was extracted, treated with RNase free DNase to eliminate contaminating genomic DNA and processed for the synthesis of cDNA and PCR (20, 21). Human specific forward and reverse primers spanning over a 497 bp fragment encoding FGFR-1 and a 625 bp fragment of β-actin cDNAs were employed (22, 23). The PCR products were separated on 1.5% agarose gel, digitally photographed and the intensity of the bands was quantified in relation to β-actin band using Molecular Analyst (V 1.5) image analysis program (Biorad Laboratories, Hercules, USA) and values were expressed as a ratio to the controls.

Statistical Analysis

Data were analysed for statistical significance using the unpaired, two-tailed Students' t-test as well as the non-parametric Mann-Whitney test, where appropriate. The data were expressed as mean ± SEM. Staining for different compartments were correlated with FEV_1 and K_{co} using Pearson's correlation analysis. Differences with $p \leq 0.05$ were considered to be statistically significant.

4.4 Results

Clinical Parameters

The clinical and lung function characteristics of all subjects included in the study are listed in Table 4.1 (16). The COPD group demonstrated an elevated residual volume (RV), whereas the CO-diffusion (K_{co}) was reduced as compared to controls ($p<0.005$). The subjects in the two groups did not differ significantly in age, total lung capacity (TLC), reversibility in FEV_1, smoking status (pack-years) or steroid use (Table 4.1).

Localization and quantification of FGF-1 and FGF-2

FGF-1 and FGF-2 were localized in bronchial epithelial and airway smooth muscle cells (ASM), epithelial cells of the mucous glands and VSM cell. In addition, FGF-1 was detected in the epithelial basement membrane (BM). Interestingly, FGF-2 was observed in the cytoplasm of bronchial surface and gland epithelium whereas in smooth muscle cells of the airway and blood vessels, the immunopositivity was nuclear. This latter, nuclear staining pattern was exclusively observed in smooth muscle cells and it was patchy so that positive nuclei were seen next to negative ones. Microphotographs showing the expression patterns of FGF-1 and FGF-2 are presented in Figure 4.2, panels A, C and E, G (non-COPD), and B, D and F, H (COPD), respectively. Video image analysis revealed that the expression levels for FGF-1 in the bronchial epithelium (Figure 4.3, panel A) were increased significantly (stained/total epithelial area: 0.32±0.04 vs. 0.20±0.03, $p<0.04$) in COPD cases as compared to non-COPD. In ASM cells no difference was found for FGF-1 (0.16±0.04 vs. 0.14±0.03, $p=0.77$).

Table 4.1 Subject characteristics and clinical parameters

	Non-COPD	COPD
FEV_1 (%pred.)	97±1.6	54±3.3*
$dFEV_1$ (% change from pred.)	3±0.6	4±0.9
FEV_1/FVC (%pred.)	100±2.1	58±2.3*
TLC (%pred.)	104±1.9	103±3.6
RV (%pred.)	117±5.4	141±10*
Kco (%pred.)	94±2.0	55±5.4*
Sex (Male/Female)	13/3	14/1
Age (years)	59±3.5	64±2.6
Smokers/ex-smokers/non-smokers	11/3/2	12/3/0
Pack-years	44±8.6	31±0.3
Steroid use (yes/no/unknown)	0/15/1	3/10/2

Abbreviations: Forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), total lung capacity (TLC), residual volume (RV), reversibility of FEV_1 after 400 μg salbutamol ($dFEV_1$) and carbonmonooxide diffusion constant (Kco) are given as percentage of predicted. * $P < 0.005$ versus non-COPD. The patients in these two groups participated in a larger project, part of which has been published previously (16, 17).

FGF-2 expression however was clearly up-regulated in bronchial epithelium of COPD cases (0.35±0.03 vs. 0.20±0.04, p<0.008, Figure 4.3, panel B) and ASM nuclei (LI ASM nuclei, 0.84±0.07 vs. 0.32±0.06, p<0.0001, Figure 4.3, panel C). The distribution of total nuclei/total ASM tissue area remained unchanged in both the groups indicating that the number of nuclei as well as the ASM area increased simultaneously, keeping the ratio equal in both groups (data not shown). Furthermore, it appeared that COPD was associated with an increase in FGF-2 expression in ASM cells with perhaps increase in their size but without their apparent proliferation.

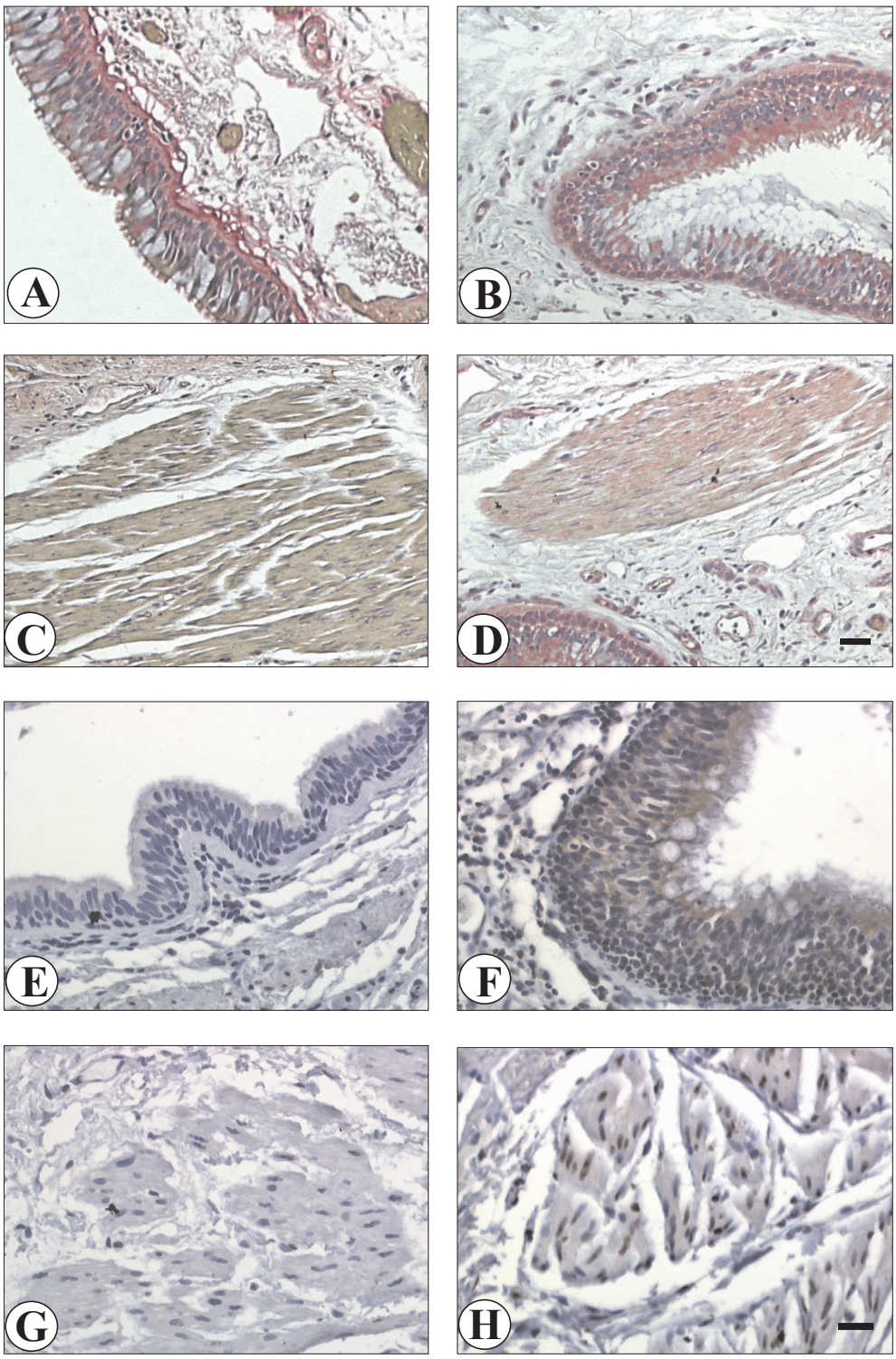

Figure 4.2 **Immunohistochemical localization of FGF-1 and FGF-2 in central airways.** Photomicrographs of central bronchial tissue sections from patients without COPD (A, C, E and G) and with COPD (B, D, F and H). Panels A and B show representative examples of FGF-1 protein staining (red new-fuchsine) in bronchial epithelium. Panels **C** and **D** show representative staining in airway smooth muscle (ASM) cells. Original magnification: x200. Panels **E** and **F** show representative examples of FGF-2 protein staining (brown 3, 3-diaminobenzidine) in bronchial epithelium. Panels **G** and **H** show representative nuclear staining in airway smooth muscle (ASM) cells. Original magnification: x400. Scale bar = 50 μm.

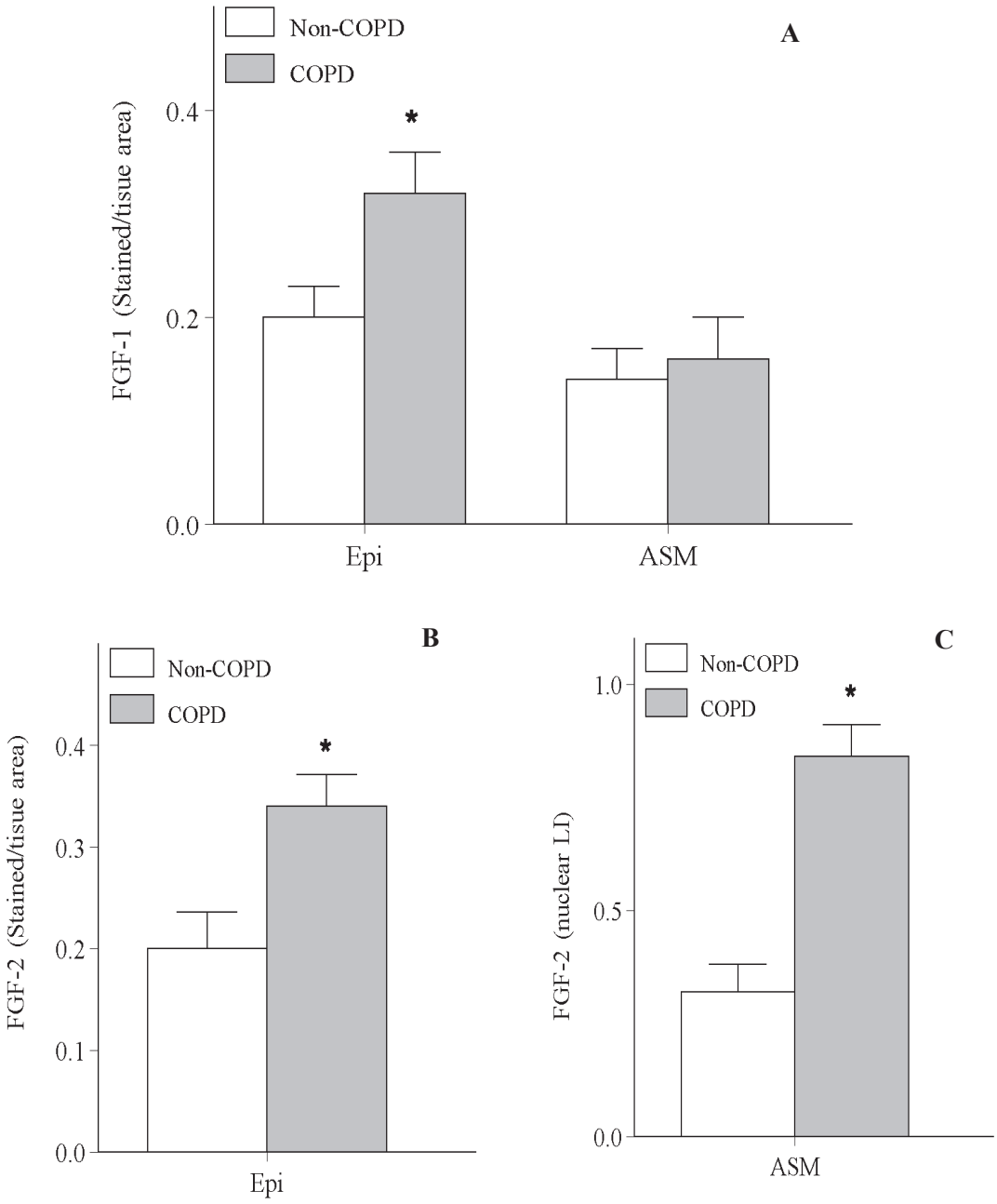

Figure 4.3 **Quantitative analysis of FGF-1 and FGF-2 expression.** Graphic representations of FGF-1 expression using video image analysis **(A)** in Bronchial epithelium (EPI) and Airway Smooth muscle cells (ASM), and FGF-2 expression **(B)** in Bronchial epithelium depicted as a ratio of stained area divided by tissue area in non-COPD (white bars) and COPD groups (gray bars). **(C)** FGF-2 expression in ASM cells presented as Labeling Index (LI) of total ASM nuclei. Values are mean ± SEM from 13-15 patients in each group. * **P** <0.05 versus the non-COPD group.

Localization and quantification of FGFR-1

FGFR-1 immunoreactivity was detected in bronchial epithelial and airway smooth muscle cells, and the endothelium and vascular smooth muscle of bronchial small vessels. Microphotographs showing the expression pattern of FGFR-1 are presented in Figure 4.4, panels A and C (non-COPD), and B and D (COPD). Graphic representations of the data as assessed by video image analysis for FGFR-1 immunostaining is shown in Figure 4.5, panel A. The expression of FGFR-1 was up-regulated in COPD in bronchial epithelium (0.21±0.03 vs. 0.08±0.02, $p<0.001$) and ASM cells (ASM/total ASM area, 0.31±0.05 vs. 0.11±0.03, $p<0.005$). Assessing the expression of both FGF-1 and FGF-2 in VSM cells using visual scoring, only FGF-2 expression levels were found to be higher in COPD as compared to non-COPD (fold increase 1.65, $p<0.01$, Figure 4.5, panel B). Elevated staining of FGFR-1 in COPD as compared to non-COPD patients was observed in smooth muscle of subepithelial microvessels (1.6 fold increase, $p<0.05$, Figure 4.5, panel B).

Bronchial airways were also stained with smooth muscle specific antibody, α-SMA (Figure 4.4, panel E) as well as with cell proliferation marker, Ki-67 (Figure 4.4, panel F). The majority of ASM and VSM cells stained positive for α-SMA in both non-COPD and COPD groups. Ki-67 immunoreactivity was mainly observed in the nucleus of basal and parabasal epithelial cells, and also in some inflammatory cells. Surprisingly, we only found very rarely an ASM cell stained with Ki-67 and this was the case in both COPD and non-COPD groups.

Correlation of FGFs and FGFR-1 expression with clinical data

Pearson's correlation of FGF-1, FGF-2 and FGFR-1 expression with clinical parameters in COPD and non-COPD patients is summarized in Figure 4.6. For FGF-1, FGF-2 and FGFR-1, we observed a significant, inverse correlation between the epithelial expression with both FEV_1 and FEV_1/FVC, and a positive correlation of epithelial FGF-1 expression and packyears ($r=0.49$, $p<0.01$). Moreover, we found significant inverse correlation of FGF-2, and FGFR-1 staining in ASM cells with both FEV_1 and FEV_1/FVC ($r = -0.71$, $p<0.0001$). Regarding the expression of FGFR-1 and its ligands, we observed a significant positive correlation with FGF-1 ($r=0.53$,

p<0.001) and with FGF-2 (r=0.64, p<0.001) in ASM. In the epithelium these values were r=0.52 (p<0.001) and r=0.64 (p<0.001), respectively. However, no significant correlation was found between FGF-1 and FGF-2 localization.

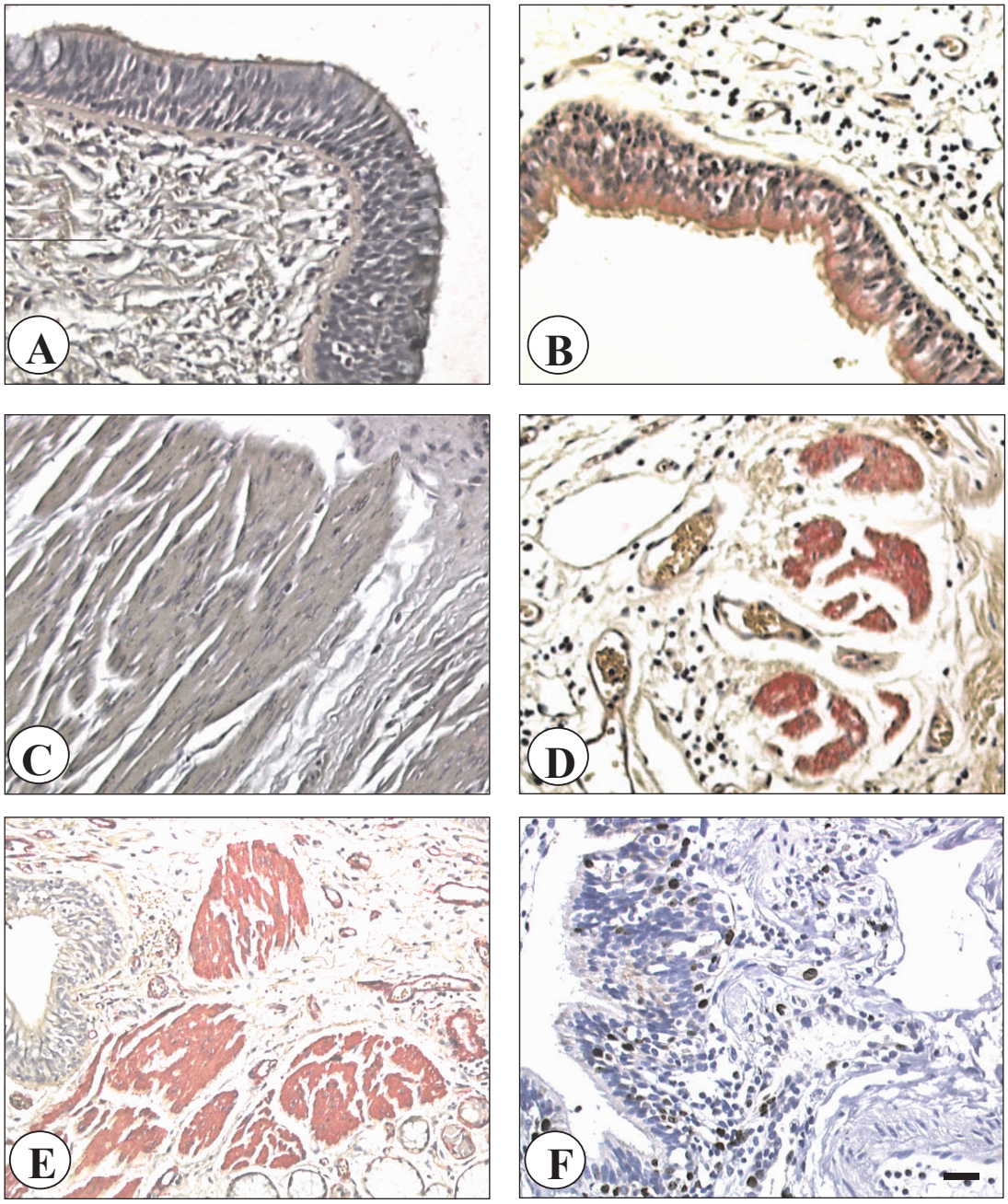

Figure 4.4 **Immunohistochemical localization of FGFR-1, α-SMA and Ki-67 in central airways.** Photomicrographs of central bronchial tissue sections from patients without COPD (**A**) and with COPD (**B**) showing FGFR-1 staining (red new-fuchsine) in bronchial epithelium. Panels **C** (non-COPD) and **D** (COPD) show representative staining in airway smooth muscle (ASM) cells. Representative staining in bronchial airways for α-SMA **(E)** and for cell proliferation marker, Ki-67 immunoreactivity **(F)** in COPD cases. Original magnification: x200. Scale bar = 50 μm.

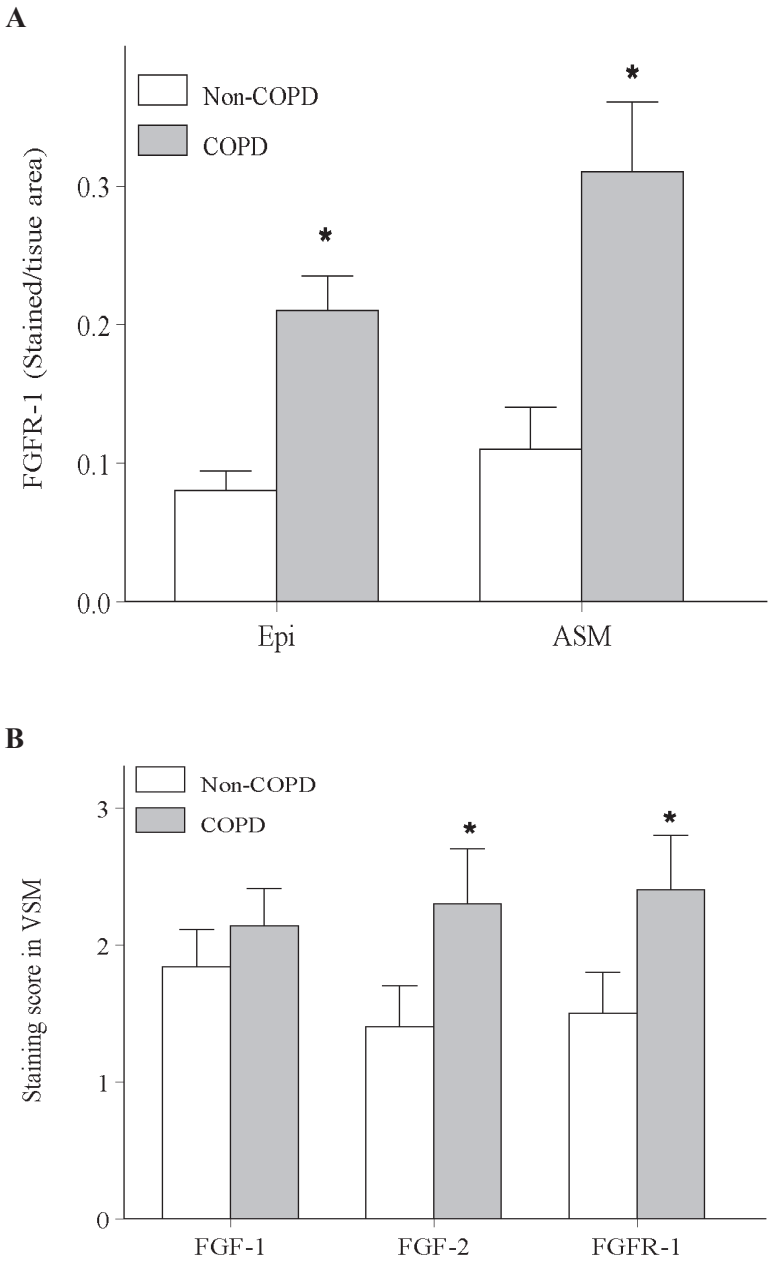

Figure 4.5 **Quantitative analysis of FGFs and FGFR-1 expression.** Graphic representations of FGFR-1 expression using video image analysis **(A)** in Bronchial epithelium (EPI) and Airway Smooth muscle cells (ASM), depicted as a ratio of stained area divided by tissue area in non-COPD (white bars) and COPD groups (gray bars). **(B)** Graphic representations of visual staining scores for FGF-1, FGF-2 and FGFR-1 (mean ± SEM) in subepithelial microvasculature (VSM) in non-COPD (white bars) and COPD groups (gray bars). * **P** <0.05 versus the non-COPD group.

Mitogenic effects of FGFs in cultured human ASM cells

In order to further investigate the role of fibroblast growth factors on airway smooth muscle remodeling, isolated human airway smooth muscle cells were stimulated *in vitro* with increasing concentrations of FGF-1 or FGF-2. Both FGF-1 and FGF-2 resulted in significantly increased cell numbers at a concentration of 10ng/ml after 48 h of incubation. Therefore, we opted for this concentration of both the growth factors in our further experiments. Figure 4.7, panel B shows the fold increase in cell number after 48 hours of stimulation with 10 ng/ml FGF-1, FGF-2 and the combination of the two over the control. Significantly increase in ASM cell numbers (fold increase) after 48 h of incubation with FGF-1 (1.37 ± 0.08, $p<0.01$) of FGF-2 (1.45 ± 0.17, $p=0.05$) or both ligands (1.42 ± 0.14, $p<0.03$) was observed.

A graphic representation of time dependent [^{3}H]-TdR uptake at a concentration of 10 ng/ml of FGF-1 or FGF-2 is presented in Figure 4.7, panel C. After 24 of stimulation, we found significantly increased [^{3}H]-TdR uptake with FGF-1 and FGF-2, but after 48 hours only with FGF-2. The combined incubation with 10 ng/ml of each FGF-1 and FGF-2 resulted in significantly increased thymidine uptake that was comparable to 10 ng/ml of FGF-2 alone. Eight hours of stimulation with either FGF-1 or FGF-2 did not result in marked increase in [^{3}H]-TdR uptake (Figure 4.7, panel C).

To examine whether human ASM cells express FGFR-1 and if this expression is regulated by FGF-1 and/or FGF-2, we performed RT-PCR on cDNA templates derived from cells treated with 10 ng/ml of FGF-1 or FGF-2 for various time-periods and compared the expression pattern with controls. FGFR-1 mRNA could be detected in ASM cells using RT-PCR for all treatments at all different time-points (Figure 4.8). A photograph showing the representative example, after agarose gel-electrophoresis with PCR products for FGFR-1 (497 bp) and β-actin (625 bp), is shown in Figure 4.8B. Both bands were analysed using appropriate image analysis software and FGFR-1/ β-actin values of FGF-1 or FGF-2 treated ASM cells at different time-points were assessed in relation to controls (Figure 4.8B). Both bands were analysed using appropriate image analysis software and FGFR-1/ β-actin values of FGF-1 or FGF-2 treated ASM cells at different time-points were assessed in relation to controls.

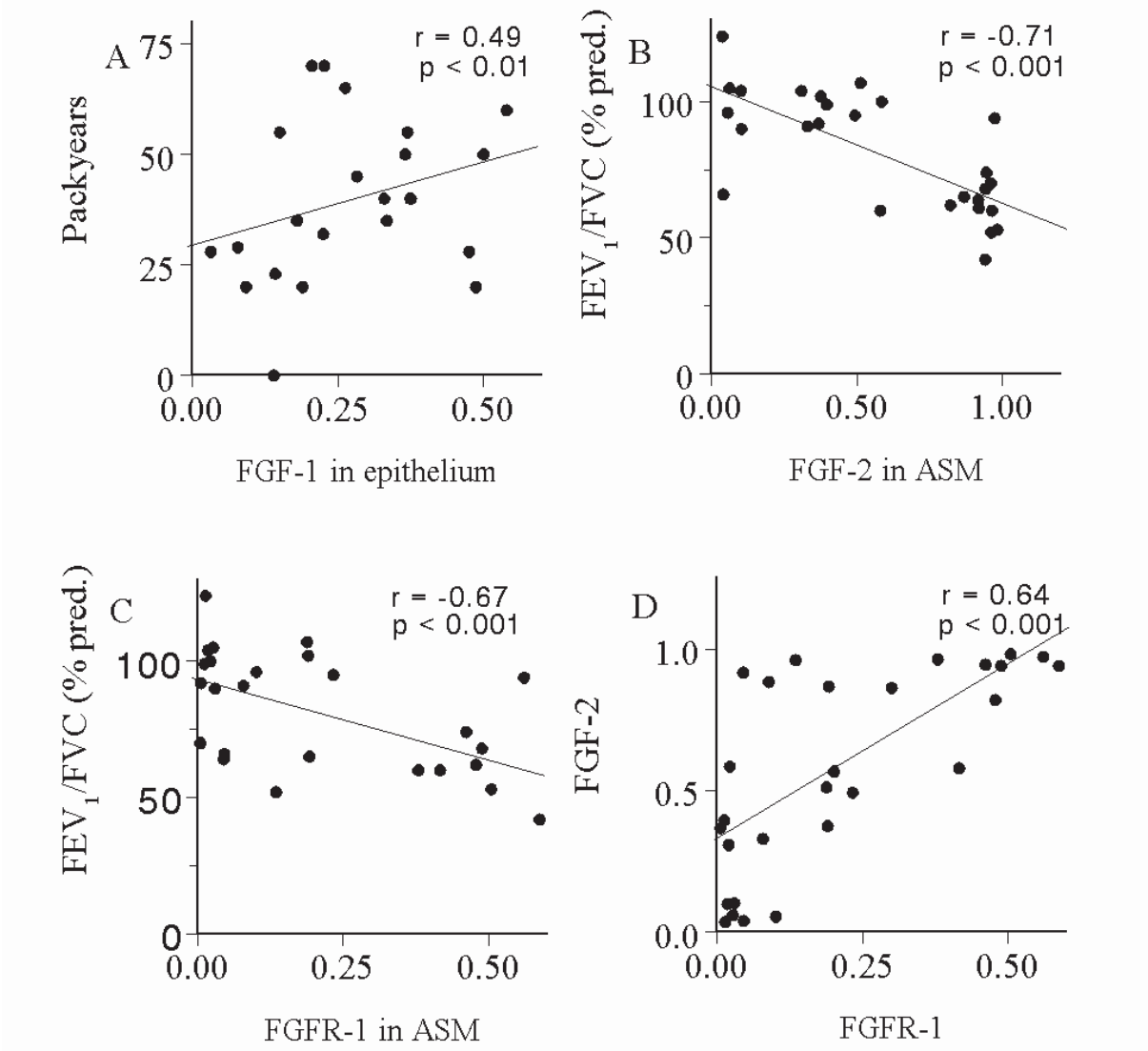

Figure 4.6 **Correlation analysis of FGFs and FGFR-1 expression.** Correlation was made for Packyears with FGF-1 in bronchial epithelium, FGF-2 in ASM with forced vital capacity and FGFR-1 and FGFR-1 in ASM with forced vital capacity. Correlation coefficient (r) was obtained using linear regression (Pearson's) analysis and significance level P value, $P < 0.05$. *Abbreviations:* Forced expiratory volume in 1 second (FEV_1), FEV_1/FVC (forced vital capacity).

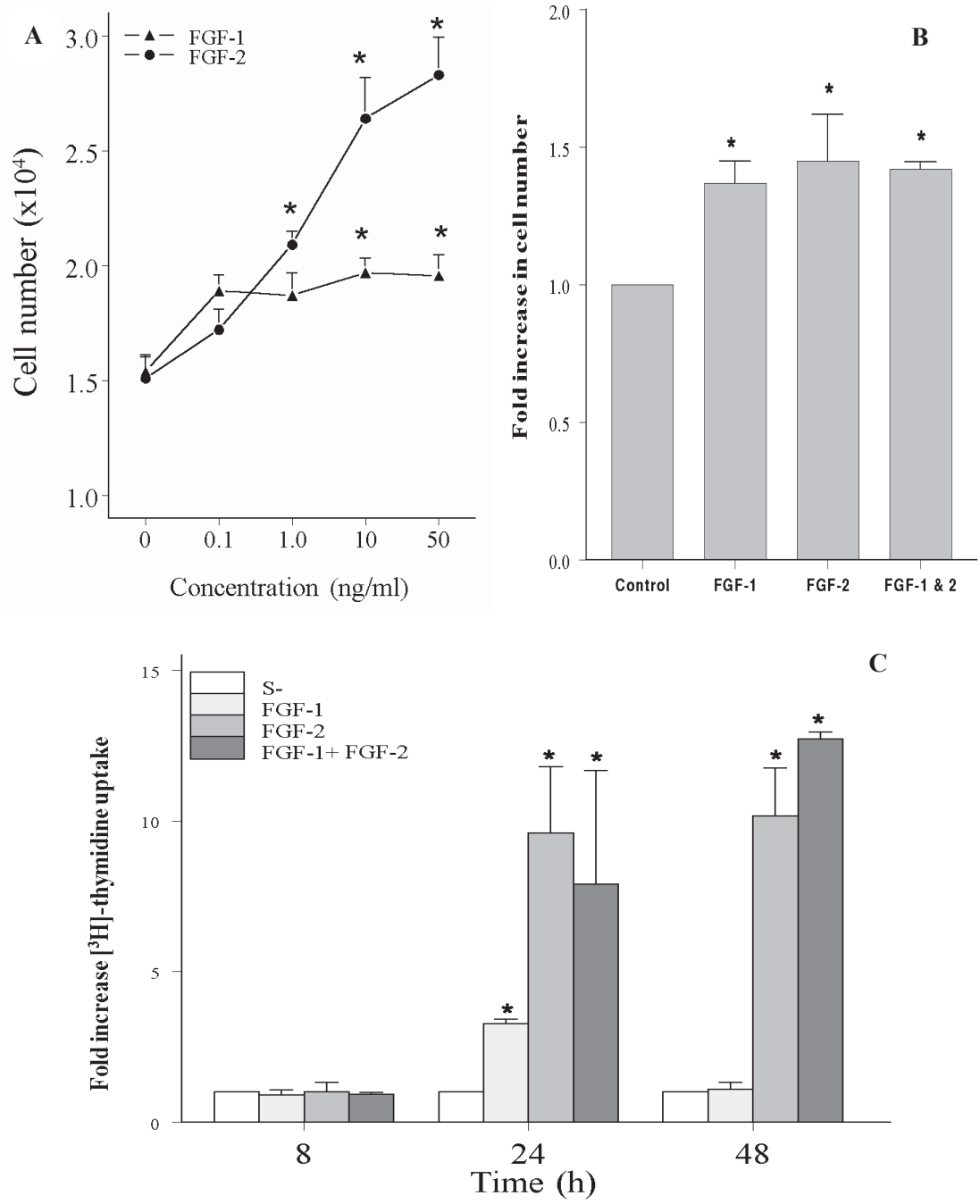

Figure 4.7 **Assessment of human ASM cell proliferation in relation to FGF-1 and FGF-2. Panel A:** A graphic representation of dose-dependent increase in cell number of human ASM cells after 48 hours stimulation with increasing concentrations of FGF-1 or FGF-2. **Panel B:** Fold induction in ASM cells relative to control after stimulation with 10 ng/ml of FGF-1, FGF-2 or a combination of both ligands for 48 h. **Panel C:** Time course of [^{3}H]-thymidine uptake in ASM cells after stimulation with 10 ng/ml of FGF-1 and FGF-2 a combination of both ligands. Data is represented as mean fold increase in relation to control from three independent experiments performed in quadruplicate. Values are mean ± SEM and ***P** <0.05 versus the control group.

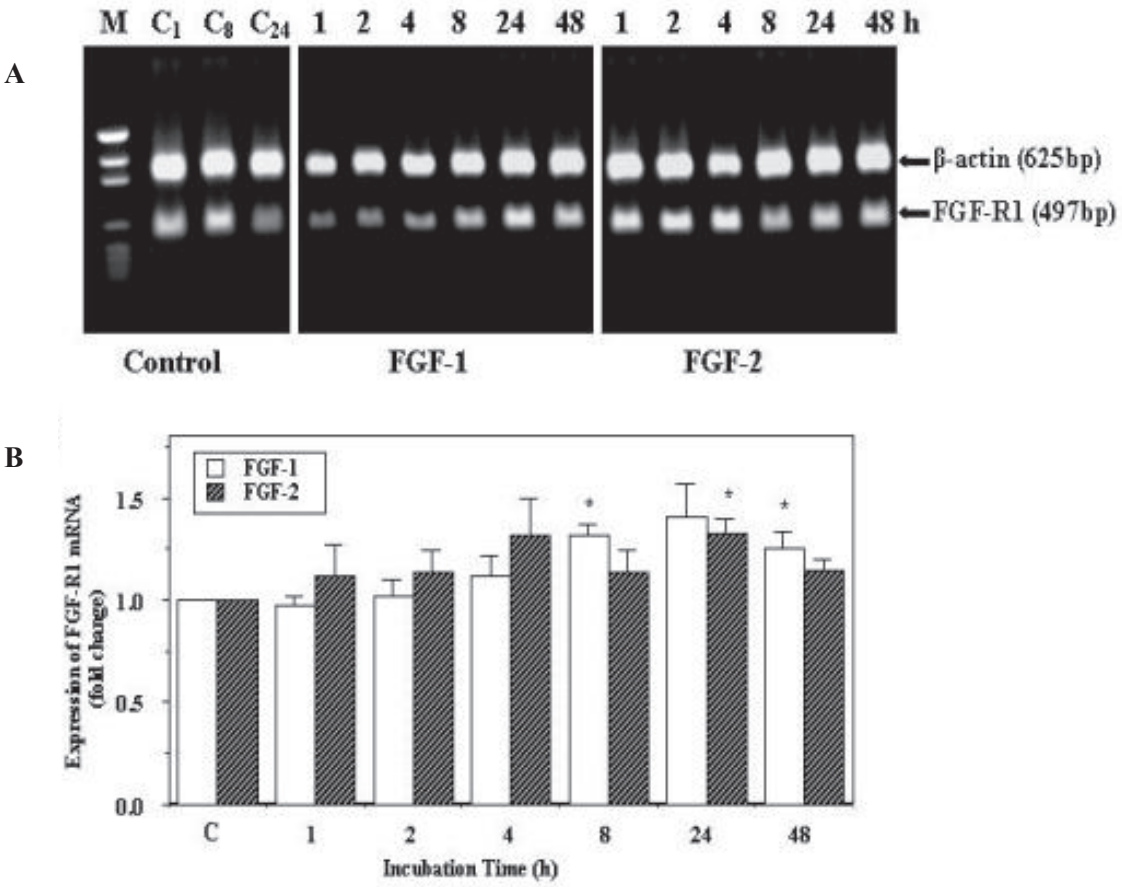

Figure 4.8 **RT-PCR analysis of FGFR-1 mRNA expression in human ASM cells.** Agarose gel electrophoresis of RT-PCR products of cDNA synthesized from human ASM cells treated with FGF-1 or FGF-2 (10ng/ml) (n=3). Representative example of an agarose gel-electrophoresis (**Panel A**) with PCR products for FGFR-1 (497 bp) and β-actin (625 bp). The different lanes marked on top denote: pGEM marker (M), Control cells at 1, 8, 24h (C_1, C_8, C_{24}), FGF-1 or FGF-2 stimulated ASM cells for 1,2, 4, 8, 24 and 48 h. Bar diagram showing quantitative analysis of FGFR-1 mRNA expression (**Panel B**). Intensity of the bands was analyzed using digital image analysis software and FGFR-1/β-actin ratio was calculated as described in the text. Values are mean±SEM from 4 independent measurements.

Stimulation with 10 ng/ml FGF-1 increased FGFR-1 mRNA expression by 1.31±0.11 fold at 8 h and by 1.23±0.12 fold at 48 h of incubation as compared to control ($p<0.05$). Whereas, FGF-2 stimulation resulted in elevated levels for FGFR-1 mRNA at 4 h (1.32±0.14 fold, $P<0.05$) and at 48 h (1.21±0.13, ns) of incubation.

4.5 Discussion

In this study we have shown that COPD is associated with an increased expression of FGF-1, FGF-2 and FGFR-1 in the bronchial epithelium and an increased expression of FGF-2 and FGFR-1 in airway smooth muscle. Correlation analysis revealed a significant inverse correlation of FEV_1/FVC with FGF-1, FGF-2 and FGFR-1 staining in the bronchial epithelium and with FGF-2 and FGFR-1 expression in airway smooth muscle. Additionally, a positive correlation of packyears with FGF-1 was found in bronchial epithelium, indicating that the degree of pulmonary FGF-1 expression is related to the amount of airway exposure to smoke. Our in vitro results indicate that FGF-1 and FGF-2 are potent mitogens for isolated human airway smooth muscle cells. Taken together, these findings strongly suggest that the FGF-FGFR system contributes to the airway remodeling.

Using video image analysis, we assessed systematically the expression of FGF-1, FGF-2 and FGFR-1 in the airways of non-COPD and COPD patients. Members of the fibroblast growth factor family FGF-1, FGF-2 and FGFR-1 are constitutively expressed in normal human lungs, particularly in bronchial epithelium, alveolar macrophages and monocytes, as well as in the intima and media of pulmonary blood vessels. Pulmonary expression patterns of FGF-1, FGF-2 and FGFR-1, found in our study are in agreement with results by Hughes and Hall (24) on the expression of these growth factors in the normal lungs. In additional, we observed FGF-1 staining and FGF-2 immunoreactivity in airway smooth muscle cells.

Several studies have commented on the importance of structural and functional abnormalities and the expression of growth factors in the bronchial airways of patients with chronic obstructive lung diseases like COPD (25-29). In asthma many growth factor/receptor systems are though to be involved in tissue remodeling, including the EGF/EGFR, TGF-β, IGF-1 and FGF/FGFR systems. The combined effects of EGF, FGF-1 and FGF-2, IGF-1 and TGF-β on epithelial cells and (myo-) fibroblasts were shown to be necessary for regulating repair of epithelial injury by induction of cellular proliferation and collagen synthesis (8, 30, 31). These same factors could however also be involved in fibrosis and tissue remodeling in asthma and possibly also in COPD (32).

Fibroblast growth factor family members are implicated in tissue remodeling in a wide variety of pathophysiological conditions including pulmonary hypertension, ischemic heart disease and interstitial lung fibrosis (12, 33-35). Barrios and co-workers (12) showed FGF-1 and FGFR-1 expression in experimentally induced pulmonary fibrosis. Becerril and colleagues showed that FGF-1 expression in the lung fibroblasts results in down-regulation of collagen synthesis and up-regulation of collagenases, which may protect against fibrosis (36). Furthermore, increased FGF-2 and FGFR-1 expression in vascular smooth muscle cells in vitro in response to vascular injury has been shown to be associated with extracellular matrix remodeling, cellular proliferation, down-regulation of collagen type I and up-regulation of collagenase, MMP-1 (37). Our findings of up-regulated FGF-1, FGF-2 and FGFR-1 expression could indicate that such compensatory mechanisms are also active in COPD, since smoking has been suggested to have a strong effect on the misbalance of proteases/anti-proteases including elastases, collagenases and extracellular matrix deposition in the lungs. Furthermore, FGF-1 and FGF-2 in the bronchial epithelium could be involved in proliferation and repair of epithelial cells after injury, which could be higher in COPD patients. This notion is supported by our findings of increased Ki-67 expression in the bronchial epithelium of COPD patients. Several authors also showed this expression in proliferating airway epithelial cells in biopsies of normal, asthma and chronic bronchitis patients (38, 39).

In the present study, we show increased FGF-2 and FGFR-1 expression but not FGF-1 in airway smooth muscle cells using immunohistochemistry. By interactively counting of ASM nuclei using video image analysis we found a highly significant increase in positive cells in COPD. Singh and colleagues have shown that increased nuclear expression of high molecular weight (HMW) FGF-2 in vascular smooth muscle and endothelium precedes arterial enlargement in response to increased arterial blood flow in vivo (34). Although the function of this FGF-2 in the cell nucleus remains unclear, this FGF-2 is believed to be targeted for translocation to the nucleus. Recently, the role of FGF-2 in the nucleus has been partly clarified, as has been reviewed in two recent reviews (40, 41). The basic FGF gene can produce at least five different isotypes: the conventional 18 kDa extracellular bFGF, as well as four additional high molecular weight forms which are predominantly nuclear in localization. All five isoforms are able to translocate to the nucleus upon activation of different cells. In the nucleus, FGF-2 can act as modulator of ribosomal gene

transcription via direct interaction of the regulatory subunit of the protein kinase CKII. Also the FGF receptors can be translocated to the nucleus, as was evidenced by a study of Stachowiak and co-workers showing co-localization of the receptor FGFR-1 and FGF-2 in the nucleus, which could indicate a novel FGFR-1 and FGF-2 functional mechanism (42). From the pattern we observed, we assume that the positivity in the nuclei was not due to an artefact but representative of specific localization of the appropriate antigen by the antibody used. In the same section some nuclei were distinctly positive, whereas, adjoining nuclei were clearly negative. Taken together the role of FGF-2 isoforms in the nucleus is very complex, but may well represent an important feature in the functional regulation.

Our ASM cell culture experiments *in vitro* indicate that FGF-2 and to a lesser extent FGF-1, are potent mitogens for airway smooth muscle cells, as was evidenced from increased [^{3}H]-thymidine incorporation. However, scarce Ki-67 positive ASM cells in COPD despite enhanced FGFs expression indicate for low turn over and untimely proliferation due to tissue damage. Our results are in accordance with previous studies on the mitogenic activity of these molecules (43, 44) and further strengthen for the role of FGFs COPD. Pearson's correlation analysis revealed significant inverse correlation of FEV_1 on the one hand with expression of FGF-1, FGF-2 and receptor FGFR-1 in bronchial epithelium, and on the other hand with FGF-2 and FGFR-1 in ASM. These findings may indicate that the expression of these molecules is related to airflow limitation. Additionally, we observed a positive correlation of epithelial FGF-1 expression and packyears in all patients, although no significant difference was observed when comparing packyears between non-COPD and COPD patients. This suggests that responses to cigarette smoke exposure are involved in epithelial cell function. We also observed highly significant correlation of FGF-1/FGFR-1 co-localization in bronchial epithelium and FGF-2/FGFR-1 in ASM cells. These findings indicate that FGF-1 and FGF-2 are differentially expressed and may regulate locally different events in the corresponding tissues.

In vivo and in vitro data indicate that smooth muscle cells, and their cross-talk with myo-fibroblasts and inflammatory cells via growth factors and cytokines, are major actors in airway remodeling due to a variety of pathophysiological conditions (36, 45-47). In line with this general picture, our findings suggest that the FGF-FGFR system contributes in airway remodeling in COPD. Taken together, our results support the notion that increased bronchial expression of FGF-1, FGF-2 and FGFR-1

in patients with COPD could participate in regulating the process of pulmonary airway remodeling. Blockade of these pathways should be considered in the development of therapeutic interventions aimed to prevent or reverse chronic airflow limitation in COPD.

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Chapter 5

Extracellular Matrix Proteins in COPD

Adapted from:

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5.1 Summary

Remodeling of airways and blood vessels is an important feature of chronic obstructive pulmonary disease (COPD), however its molecular mechanisms are poorly understood. We examined the expression patterns of various extracellular matrix (ECM) components, including collagens (total collagen, subtypes collagen I, III and IV), fibronectin and laminin in bronchi from smokers with COPD (FEV_1 ≤75% pred.; n=15) and without COPD (FEV_1 ≥85% pred.; n=16). Immunohistochemical staining results were assessed by a validated visual scoring method (grade 0-4). Staining for ECM components was observed in the surface epithelial basement membrane (SEBM), and within the interstitium and vessels of the lamina propria and adventitia of airways. In COPD, total collagen was increased in the SEBM ($p<0.01$) at sites of intact bronchial epithelium, but was not changed in the interstitial space and vessels of the airway lamina propria and adventitia. Deposition of collagen I and III, however, was enhanced in the SEBM both at sites of damaged and of intact surface epithelium ($p<0.05$), lamina propria ($p<0.02$) and bronchial adventitia ($p<0.05$) in COPD. In COPD, fibronectin was increased in vessels of the lamina propria ($p<0.05$) and laminin in airway smooth muscle ($p<0.01$) and the microvasculature ($p<0.05$). FEV_1 values inversely correlated with collagens in the SEBM, fibronectin in bronchial vessels and laminin in the ASM. We conclude that smokers with COPD exhibit increased bronchial deposition of collagens I and III, fibronectin and laminin as part of the airway remodeling process in COPD.

5.2 Introduction

Chronic obstructive pulmonary disease (COPD) is a global health problem with increasing morbidity and mortality. One of the major causal factors is tobacco smoking, but of all smokers, only 10-20 percent develop COPD (1). Pathological features of COPD include thickening of airway walls, probably as a result of ongoing chronic inflammatory processes with an influx of neutrophils, macrophages and T-lymphocytes (2). The resultant changes in the airway wall in COPD include hyperplasia of subepithelial (myo-)fibroblasts and airway smooth muscle cells (3, 4).

Previous studies on the pathology of COPD have focused on alterations in small airways and parenchyma, where an infiltration of $CD8^+$ T cells and macrophages, a loss in the number of alveolar-bronchiolar attachments and ECM (emphysema) with consequent loss of elastic recoil, and alveolar-peribronchial wall fibrosis with increased deposition of ECM proteins have been demonstrated (5-8). Thus far, few studies of COPD have focused on the larger airways (9-11). Bronchial epithelial loss and changes in large airway dimensions are found in COPD (3, 10, 12). Tiddens and co-workers reported that the thickness of the wall area internal to the airway smooth muscle was increased in COPD, and that this increase correlated inversely with the FEV_1/FVC ratio, but these authors found no difference with respect to the airway smooth muscle mass (10).

Thickening of the surface epithelial basement membrane (SEBM), subepithelial fibrosis and the deposition of extracellular matrix proteins in the lamina propria are key features in asthma (13, 14). In COPD, however, changes in thickness of the SEBM and fibrosis of the mucosal lamina propria are less pronounced (15). Recent studies have indicated that the SEBM thickness in bronchial biopsies from smokers with chronic bronchitis was similar to that in normal subjects, unless features of asthma such as hyperresponsiveness or corticosteroid sensitivity were present as well (11, 15, 16). However, it has not been investigated in detail whether the composition of the SEBM is unchanged in COPD, and in addition, the lamina propria and adventitia may be altered.

We postulated that alterations in total or relative content of extracellular matrix proteins such as collagens, including subtypes I, III and IV, fibronectin, laminins and proteoglycans in the various compartments of the bronchial wall (SEBM, lamina propria, and bronchial adventitia and smooth muscle) are present in the airways of (ex-) smokers with COPD. In this study we investigated the localization and distribution pattern of various ECM markers in bronchial tissue from (ex-)smokers with or without COPD. Taken together, our results indicate that COPD is associated with increased deposition of ECM components in the bronchial airway wall. This may contribute to airway remodeling and airflow limitation.

5.3 Materials and methods

Bronchial tissue from lobectomy or pneumonectomy of current and ex-smokers, who underwent surgery for lung cancer, was obtained from the archive of the Pathology Departments of the Leiden University Medical Center (LUMC, Leiden, The Netherlands) and Southern Hospital (Rotterdam, The Netherlands), after approval of the study by the Medical Ethics committee of LUMC. All lung tissues were expanded by an injection syringe using 10 % phosphate-buffered formalin, and fixed for approximately 24 hours after which the tissues were further processed for embedding in paraffin and immunohistochemical staining. Samples of bronchial airways, located as far away as possible from the tumour were chosen for the study. Based on lung function outcome (see below), patients were assigned to the COPD and non-COPD groups (17-19). The patients in these two groups participated in a larger research project, part of which has been published previously (19, 20).

COPD group: Fifteen subjects were assigned to this group on the basis of the following parameters: forced expiratory volume in one second (FEV_1) <75% of predicted value before bronchodilatation, FEV_1/FVC ratio <75%, a reversibility in FEV_1 ≤12% of predicted after 400 μg inhaled salbutamol, and a transfer factor for carbon monoxide (diffusion capacity) per litre alveolar volume (K_{co}) ≤80% of predicted value (21).

Non-COPD group: Sixteen subjects were assigned to this group based on the basis of the following data: FEV_1 >85% of predicted before bronchodilatation, FEV_1/FVC ratio >85%, and reversibility in FEV_1 ≤12% of predicted after 400 μg salbutamol inhalation. In order to exclude accompanying lung disease leading to a restrictive lung function, it was required that the total lung capacity (TLC) of each subject was over 80% of the predicted value (21).

Clinical data of all patients were examined for possible co-morbidity and medication use. All patients were free of symptoms of upper respiratory tract infection and none received antibiotics perioperatively. None of the patients received glucocorticosteroids in the three months prior to operation, but four patients received oral glucocorticosteroids perioperatively. After the selection based on lung function, all the lung tissues used for this study were checked histologically using the following exclusion criteria: (i) presence of tumour, (ii) presence of poststenotic pneumonia, (iii) fibrosis of lung parenchyma, and (iv) obstruction of the main bronchus (17, 18).

Pulmonary Function Tests

All pulmonary function tests were performed within 3 months prior to surgery. FEV_1 and forced vital capacity (FVC) were measured by spirometry, TLC and residual volume with the closed circuit helium dilution test and the K_{co} using the single breath-holding technique, as described by Quanjer et al. (21). Lung function data and other patient characteristics are shown in Table 5.1.

Total collagen staining

The total collagen fibers in bronchial tissue specimens were stained with Picro-sirius Red F3BA (22). Tissue sections of 4 μm thickness were treated with 0.2% aqueous phosphomolybdic acid and incubated in 0.1% Picro-sirius Red. Before dehydration, the slides were treated with 0.01N HCl and mounted. Slides were visualized under light microscope and collagen content was assessed using the same visual scoring method used for the analysis of the immunohistochemistry data (see below).

Immunohistochemistry and quantification

Sections of paraffin-embedded lung tissue were cut at 4 μm, mounted on Super Frost Plus® microscopic slides (Menzel-Gläser, Braunschweig, Germany) and processed for immunohistochemistry. Serial sections were used to detect the staining of collagen I, III, IV, fibronectin and laminin β2 employing immunohistochemistry. Sections were deparaffinized and rehydrated prior to incubation with specific purified mouse monoclonal antibodies. Anti-human mouse monoclonal antibodies against collagen IV, fibronectin and laminin were purchased from NeoMarkers (Fremont, USA), collagen I from Sigma (St Louis, USA) and collagen III from Biogenex (San Ramon, USA), respectively. To block non-specific second antibody binding, sections were preincubated with 10% normal goat serum diluted in 5% bovine serum albumin in phosphate buffered saline (5% BSA/PBS, pH = 7.4). Subsequently, sections were incubated overnight at 4 °C with primary antibodies against collagen I (1:150 v/v) or III (undiluted), fibronectin (1:500 v/v) and laminin β2 (1:150 v/v), or for 1 hour at room temperature in case of collagen IV (1:150 v/v). Immunostainings were performed after antigen retrieval by 0.1% protease treatment in PBS for 10 minutes at 37^0C or in case of collagen I by boiling in citrate buffer (10 mM citrate buffer, pH = 6.0) for 10 minutes in a microwave oven. Incubation for 30 minutes with secondary

biotinylated anti-immunoglobulins (Multilink®, 1:75 dilution, Biogenex, San Ramon, USA) and tertiary complex of peroxidase-conjugated streptavidin at a dilution of 1:50 were used to enhance the detection sensitivity. Colour was developed using 0.025% of 3,3-diaminobenzidine (Sigma, St Louis, USA) in 0.01 mol/L PBS, containing 0.03% H_2O_2. Positive controls consisted of human breast carcinoma and placental tissue. Negative controls were not incubated with primary antibody. The optimal dilution for all antibodies was identified by examining the intensity of staining obtained with a series of dilutions: the optimum concentration resulted in specific and easily visible signal on paraffin sections of control specimens. Slides were counterstained with Mayer's hematoxylin, mounted and studied light-microscopically.

A visual scoring method was applied. For this purpose all tissues were analysed in a blinded fashion in random order by two independent observers, who were unaware of the clinical data of the case under study. Quantitative analysis was performed using a validated, arbitrary visual scale with grading scores of 0, 1, 2, 3 and 4 representing none, weak, moderate, intense and very intense staining, respectively (17, 18, 23, 24). We quantified the staining pattern of ECM proteins in the SEBM and subdivided the staining for sites where the bronchial epithelium was totally lost and the SEBM was denuded or not. Furthermore, the interstitial staining of the bronchial lamina propria and adventitia was assessed. Moreover, the staining pattern within either the microvasculature bronchial lamina propria or the adventitia was measured. The intensity of laminin expression in the ASM area was quantified. We also examined errors within and between observers by correlating the expression scores using Pearson's analysis and found a very high correlation of 0.8 to 0.9.

Statistical Analysis

Data were analysed for statistical significance using the unpaired, two-tailed Students' t-test as well as the non-parametric Mann-Whitney test, where appropriate. The expression data for ECM proteins were expressed as mean ± SEM. Furthermore, ECM proteins staining for different compartments were correlated with FEV_1 using Pearson's correlation analysis. The individual collagen subtype values were correlated with the total collagen staining and with each other to evaluate co-localisation. Differences with $p \leq 0.05$ were considered to be statistically significant.

5.4 Results

Clinical Parameters

The clinical and lung function characteristics of all subjects included in the study are listed in Table 5.1. The COPD group demonstrated an elevated residual volume (RV), whereas the K_{co} was reduced ($p<0.005$). The subjects in the two groups did not differ significantly in age, TLC, reversibility in FEV_1, smoking status (pack-years) or steroid use (Table 5.1).

Localization and quantification of extracellular matrix proteins

We investigated the localization of extracellular matrix proteins in the bronchial airways (Figures 5.1 and 5.2). ECM proteins were systematically assessed in the following sites: the surface epithelial basement membrane (SEBM), the connective tissue of the lamina propria and adventitia of the bronchial airway and in the bronchial blood vessels. We observed staining for collagen IV, fibronectin and laminin within the SEBM relatively more towards the apical side whereas collagen I and III were localized more towards the lamina propria in the reticular layer. Within vessel walls, staining for fibronectin was found in the (neo-)intima, for collagen IV and laminin in the medial and collagen I and III in the adventitial layer. In addition, laminin was immuno-localized at the apical side of the bronchial epithelium and in the airway smooth muscle (ASM) cell layer.

Representative examples of collagen staining in non-COPD (A, C, E and G) and COPD (B, D, F and H) samples are depicted in Figures 5.1 and 5.2. We quantified the staining pattern of ECM proteins in the SEBM and subdivided the staining for sites where the bronchial epithelium was damaged or not. All investigated ECM proteins were significantly increased at sites of epithelial denudation (Figure 5.3A-F, $p<0.01$). We observed more intense staining for total collagen in the SEBM at sites of intact epithelium in subjects with COPD (1.5 fold increase, $p <0.05$, Figure 5.3A). Figure 5.3B demonstrates that collagen I deposition is increased in COPD as compared to non-COPD patients in the SEBM at the areas of intact epithelium (2.3 fold increase, $p < 0.001$) and damaged bronchial epithelium (1.6 fold increase, $p < 0.01$), lamina propria and bronchial adventitia, (1.9 fold increase each, $p < 0.001$).

Table 5.1 Subject Characteristics

Case	Sex	Age	FEV_1	FEV_1/FVC	TLC	RV	Pack-years	Steroids
Non-COPD								
1	M	72	98	104	105	122	65	n
2	M	67	102	99	112	134	29	n
3	F	73	91	91	107	125	50	n
4	M	46	109	104	97	87	23	n
5	M	58	96	92	110	121	70	n
6	M	57	94	90	95	87	35	n
7	M	86	86	96	106	140	70	n
8	M	64	93	95	110	152	20	n
9	M	28	99	102	104	143	0	n
10	M	51	97	107	99	92	0	n
11	M	38	100	94	106	100	28	n
12	M	52	100	100	103	121	20	n
13	F	58	100	105	90	90	28	n
14	M	69	110	124	100	102	u	u
15	F	61	94	107	119	142	u	n
16	M	61	86	94	95	119	u	n
Mean ± SEM		**59 ± 3.5**	**97 ± 1.6**	**100 ± 2.1**	**104 ± 1.9**	**117 ± 5.4**	**44 ± 8.6**	
COPD								
17	M	77	73	70	103	110	25	n
18	M	71	69	64	115	129	u	n
19	M	72	37	42	136	229	50	y
20	M	60	75	66	123	155	45	n
21	M	53	44	70	89	137	32	n
22	M	65	52	60	112	169	55	n
23	M	55	56	68	99	131	40	n
24	M	55	45	60	u	u	35	y
25	M	45	75	74	97	97	u	u
26	M	61	49	62	130	223	20	y
27	M	65	69	71	116	152	20	n
28	M	57	47	53	111	170	55	n
29	F	78	62	61	95	105	60	n
30	M	71	45	52	114	171	u	n
31	M	77	67	72	104	128	u	u
Mean ± SEM		**64 ± 2.6**	**54 ± 3.3**	**58 ±2.3**	**103 ± 3.6**	**141 ± 10**	**31 ± 0.3**	
P value		**0.239**	**0.0001**	**0.0001**	**0.099**	**0.008**	**0.312**	

Abbreviations: Forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), total lung capacity (TLC), and residual volume (RV), are given as percentage of predicted. M = Male, F = Female, Pre-operative steroids use (y/n/u, yes/no/unknown) P values are given as COPD versus non-COPD. The patients in these two groups participated in a larger research project, part of which has been published previously (19, 20).

Figure 5.3C indicates that in COPD patients collagen III staining is elevated in the SEBM at sites of intact and damaged epithelium (1.5 and 1.4 fold increase, $p < 0.01$, respectively). Furthermore, at fibrotic sites of lamina propria (1.4 fold increase, $p < 0.05$) and adventitia (1.3 fold increase, $p < 0.05$) of the airway wall the collagen III staining is also increased (Figure 5.3C). Collagen IV protein, however, remained

unaltered, irrespective of the presence of COPD (Figure 5.3D). Fibronectin deposition was higher in intima and (neo-)intima including endothelial cells of bronchial blood vessels in COPD (Figure 5.3E). Laminin staining was more intense in the ASM layer (1.5 times, $p < 0.01$) and small vessels in the lamina propria (1.3 times, $p < 0.01$, Figure 5.3F). No other staining differences were observed between samples from subjects with and without COPD.

Correlation of ECM proteins with clinical data

Pearson's correlation of ECM components with FEV_1 values (% predicted) in all COPD and non-COPD patients is summarized in Figure 5.4. We observed a significant inverse correlation with FEV_1 of the following parameters: total collagen staining in the SEBM underneath intact epithelium ($r = -0.47$, $p<0.01$); collagen I staining in SEBM at sites with damaged epithelium ($r = -0.61$, $p<0.01$); connective tissue of the bronchial adventitia ($r = -0.67$, $p<0.001$, Figure 5.4A) and of the lamina propria ($r = -0.53$, $p<0.01$) In the same regions similarly inverse correlation was found between collagen III and FEV_1 ($r = -0.40$, -0.42 and -0.48, $p<0.01$, Figure 5.4B). Figure 5.4C illustrates that fibronectin is also inversely correlated with FEV_1 values in endothelium ($r = -0.51$, $p<0.01$). Moreover, in ASM we found a significant inverse correlation between FEV_1 values and laminin ($r = -0.61$, $p<0.001$, Figure 5.4D). When considering co-localization of total collagen with subtypes for collagen I, III and IV, we found a significant correlation between total collagen and collagen III in the SEBM at both damaged ($r = 0.62$, $p<0.001$) and intact epithelium ($r = 0.63$, $p<0.001$). No significant correlation was found between total collagen and collagen I and IV localization.

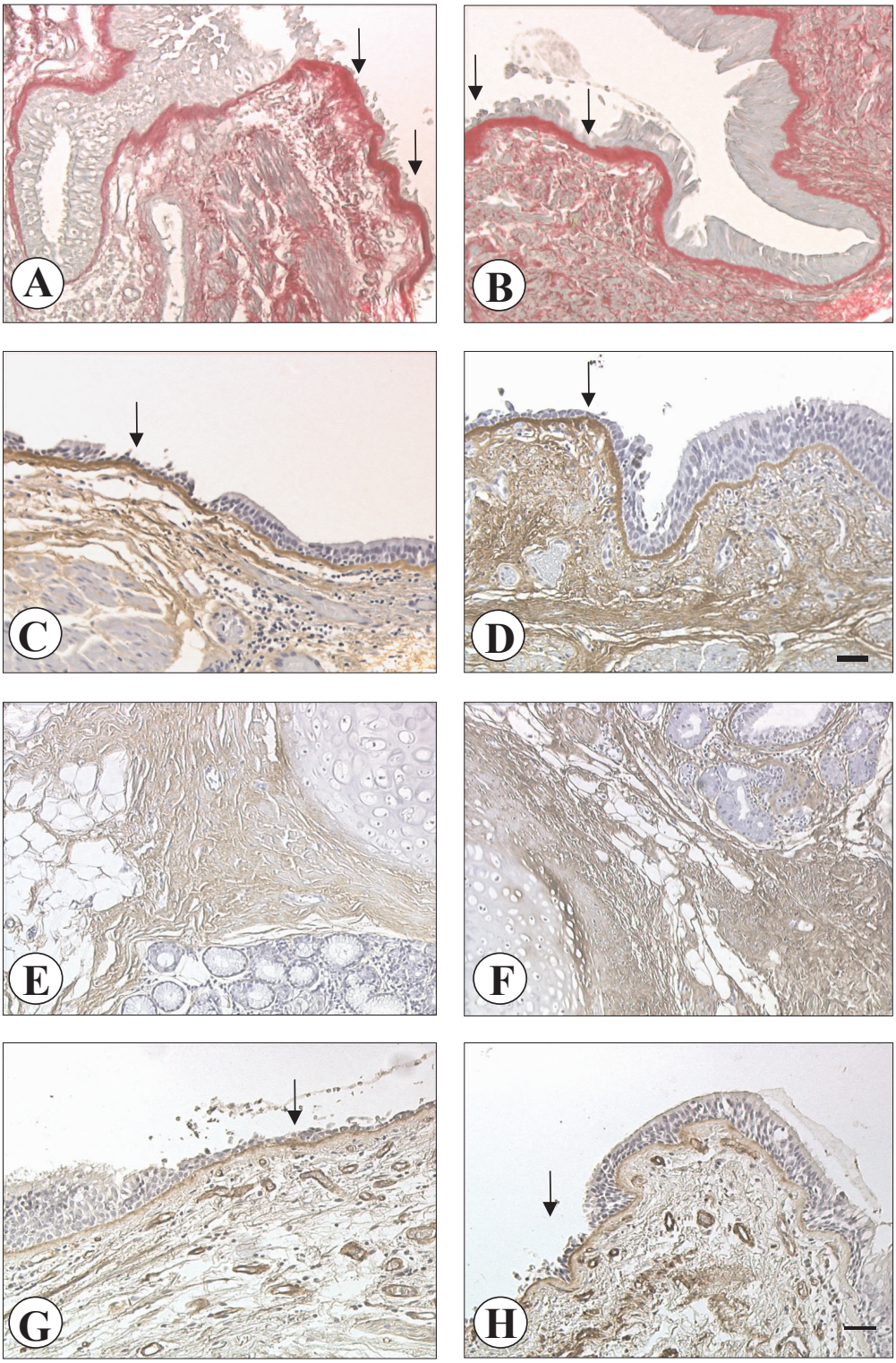

Figure 5.1 **Photomicrographs of bronchial tissue sections from patients without COPD (A, C, E and G) and with COPD (B, D, F and H).** Panels **A** and **B** show total collagen staining (Sirius-Red staining) in bronchial airway walls. Panels **C** and **D** show staining for collagen I in surface epithelial basement membrane (SEBM) and lamina propria. Panels **E** and **F** show collagen III protein staining in bronchial adventitial layer with bronchial vessels. Panels **G** and **H** show collagen IV staining in lamina propria. Arrows indicate sites of damaged bronchial epithelium. Counterstained with hematoxylin. Original magnification: x200. Scale bar = 50 μm.

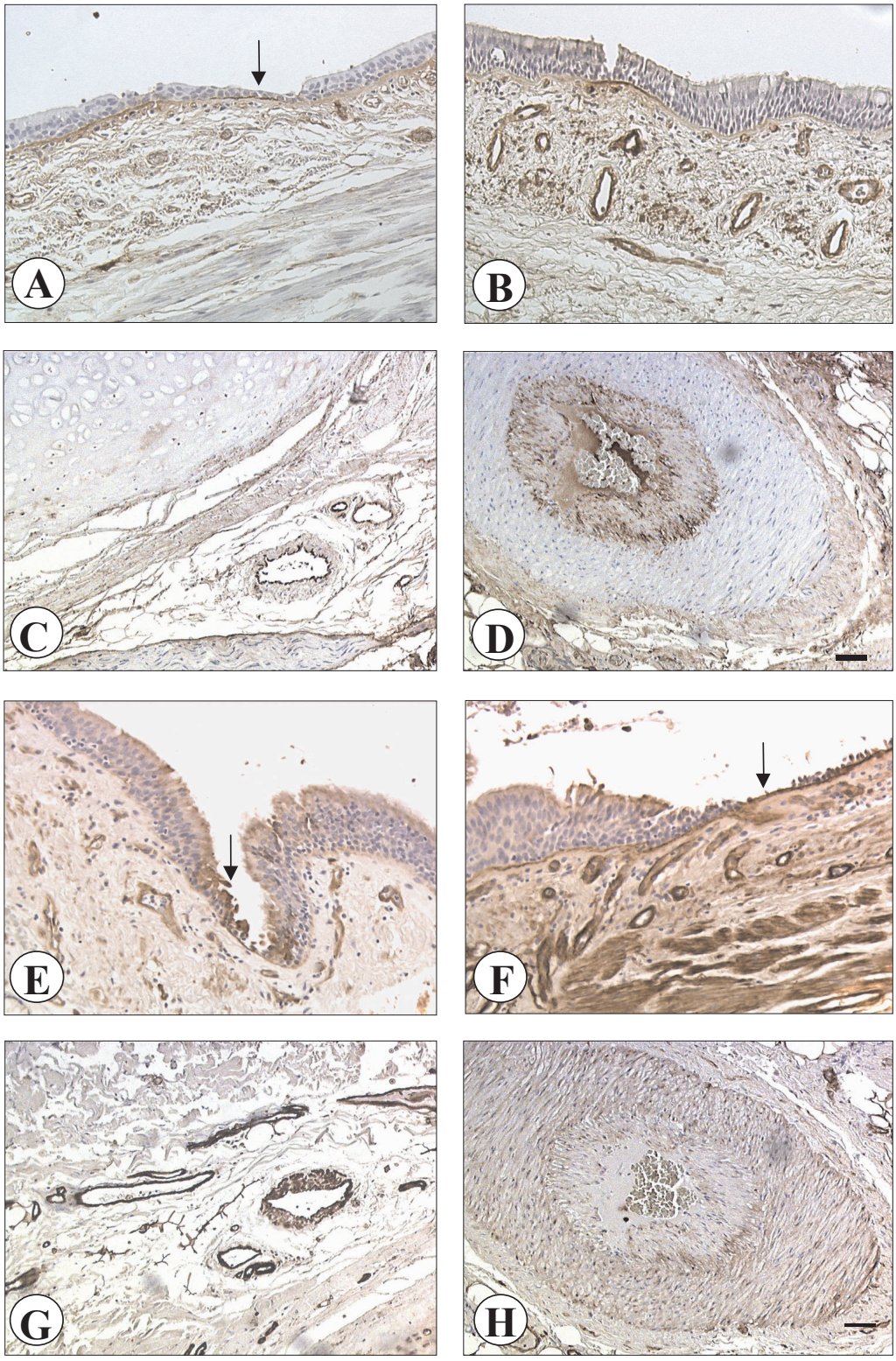

Figure 5.2 **Photomicrographs of bronchial tissue sections from patients without COPD (A, C, E and G) and with COPD (B, D, F and H).** Panels **A** and **B** show fibronectin staining in bronchial lamina propria and panels **C** and **D** in vasculature. Panels **E** and **F** show laminin protein staining in the lamina propria and panels **G** and **H** in the adventitial layers with bronchial vessels. Arrows indicate sites of damaged bronchial epithelium: Counterstained with hematoxylin. Original magnification: x200. Scale bar = 50 μm.

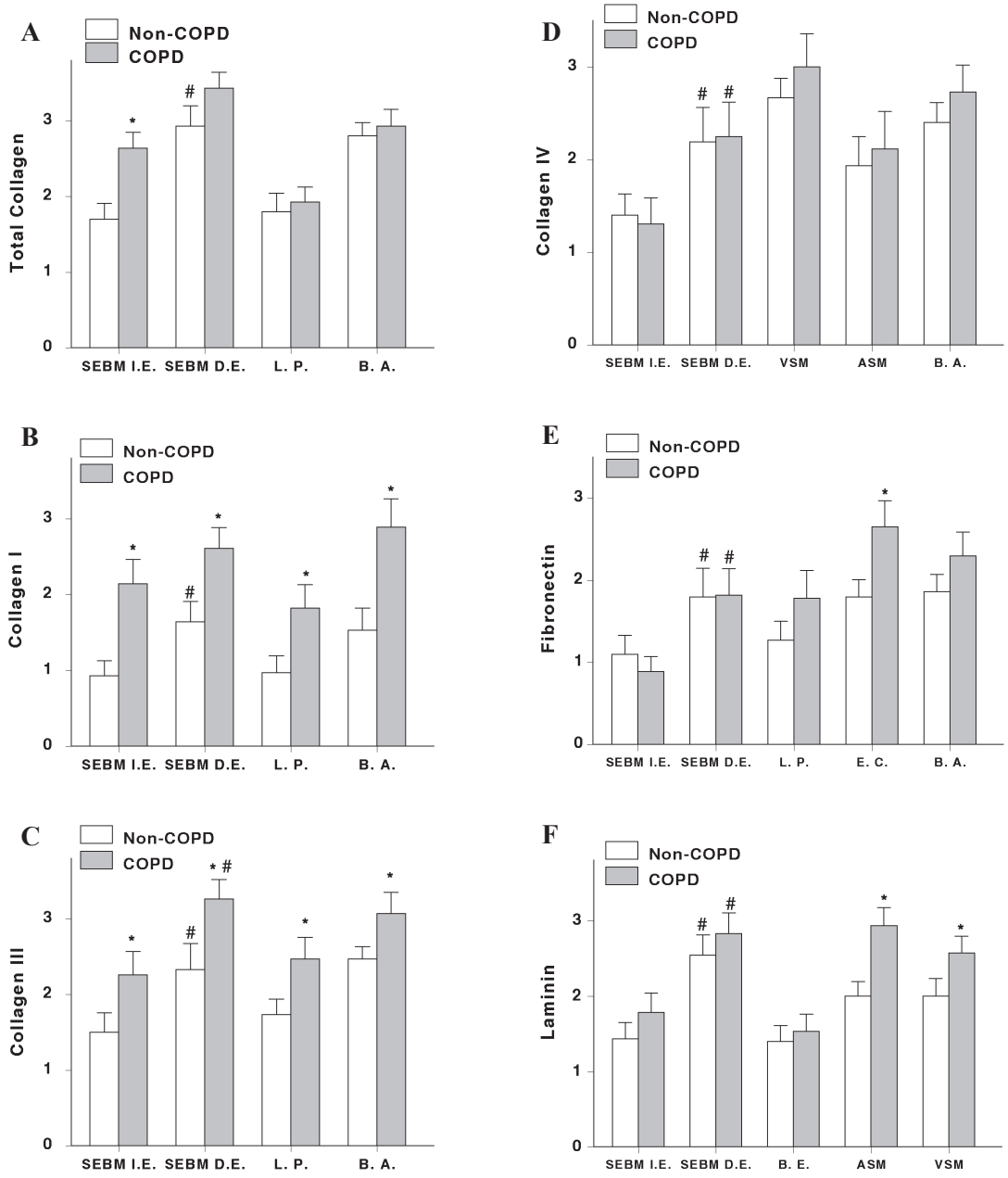

Figure 5.3 **Graphic representations of extracellular matrix proteins (mean ± SEM) using visual scoring**; **(A)** for total collagen, **(B)** for collagen I, **(C)** for collagen III, **(D)** for collagen IV, **(E)** for fibronectin and **(F)** for laminin. Abbreviations: surface epithelial basement membrane (SEBM), bronchial epithelium (B. E.), bronchial epithelium damaged or intact (D. E. or I. E.), lamina propria (L. P.), bronchial adventitia (B. A.) airway and vascular smooth muscle cells (ASM and VSM) and endothelial cells (EC). Staining score for non-COPD (white bars) and COPD groups (gray bars) are given. # **P** <0.05 SEBM scores of damaged versus undamaged bronchial epithelium. * **P** <0.05 versus the non-COPD group.

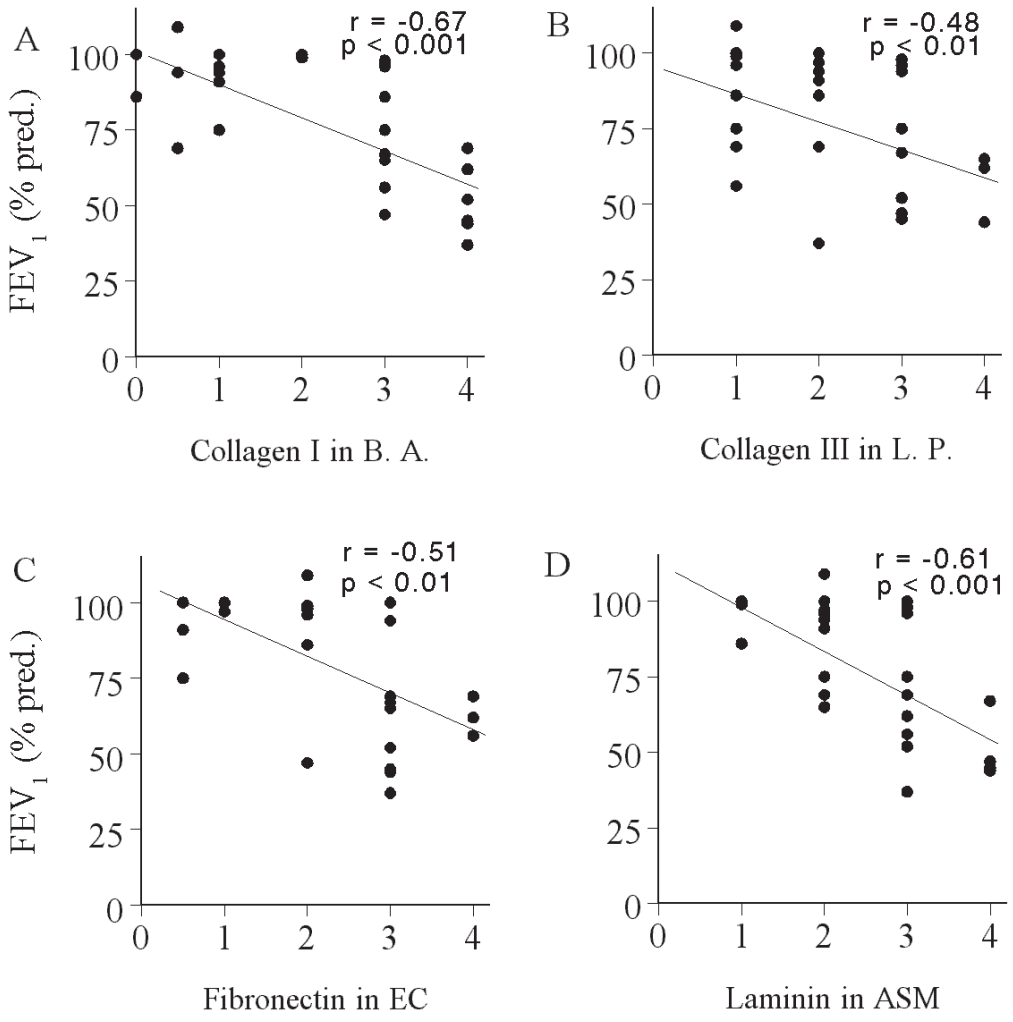

Figure 5.4 Correlation with FEV_1 (% predicted) of total collagen in SEBM with undamaged bronchial epithelium (***A***), collagen III in the lamina propria (***B***), fibronectin in endothelial cells (EC, ***C***) and laminin in VSM (***D***) of the combined patient groups (non-COPD and COPD). Correlation coefficient (r) was obtained using linear regression (Pearson's) analysis and significance level P value, $P < 0.05$.

5.5 Discussion

In this study we showed that COPD is associated with an increased bronchial deposition of collagens I, III, IV, fibronectin and laminin. ECM proteins were observed in SEBM, lamina propria and adventitia of the bronchial walls and vasculature. We found that ECM protein deposition is increased in the SEBM at sites of damaged bronchial epithelium in all patients. In COPD patients, total collagen and predominantly collagens I and III subtype were further increased as compared to controls, while bronchial vessels showed increased deposition of fibronectin and laminin. FEV_1 values inversely correlated with collagens in the SEBM, fibronectin in bronchial vessels and laminin in the ASM. Taken together, these findings strongly suggest that deposition of ECM components contributes to the airway remodeling of COPD.

An identical localization pattern of the various investigated ECM makers in the cartilaginous bronchial wall was present in our patients groups, which is in agreement with earlier reports describing their presence in the bronchial airways of asthmatics (13, 25-28). Several previous reports have demonstrated structural changes with fibrosis and deposition of ECM proteins as well as loss of elastic recoil in peripheral airways and lung parenchyma of COPD patients (29-31). Inflammation, with influx of $CD8^+$ T-cells in peripheral airways and accumulation of macrophages has been reported (6, 32). Peribronchiolar and septal fibrosis are also found whereas alveolar extracellular matrix deposition is decreased in emphysema (5, 6, 32). Our results demonstrate that COPD is also associated with changes in extracellular matrix protein deposition of larger airways. In asthma, SEBM thickening is prominent, as is the deposition in large airways of various ECM proteins, including collagens, fibronectin, laminins and proteoglycans in epithelial SEBM, subepithelial layers and bronchial vasculature (28, 33-35). In COPD, however, the few previous reports that are available have indicated that SEBM thickness remains unchanged, unless features of asthma such as hyperresponsiveness or corticosteroid sensitivity were present (3, 16, 28). However, we show here that the staining of total collagen, collagen I and III in SEBM is more intense in COPD as compared to controls. Furthermore, all investigated extracellular matrix proteins were upregulated at sites where the epithelial lining was damaged. These findings support the hypothesis of involvement

of the bronchial epithelium and subepithelial (myo-)fibroblasts in damage and repair processes with tissue remodeling. Recent studies based on in vitro co-culture experiments indicate that effects of growth factors such as epidermal growth factor (EGF), fibroblast growth factors (FGF-1 and FGF-2) and transforming growth factor beta 1 (TGF-β_1) on epithelial cells and (myo-)fibroblasts are necessary to mediate repair of epithelial injury by induction of cellular proliferation and collagen synthesis (36-39). The above mechanisms that were found *in vitro* could possibly also play a role in tissue remodeling and fibrosis during COPD.

We also investigated the deposition of ECM proteins in the bronchial vasculature of the bronchial lamina propria and adventitia. We show that COPD is associated with more deposition of collagen III and laminin in vascular media and adventitia, and with fibronectin in endothelial cells and also neo-intima of small muscular vessels. We and others have previously shown that structural changes to the pulmonary vasculature, including intimal and medial thickening with VSM hypertrophy and lumen narrowing, occur in COPD (19, 23, 40-42). We described that in the peripheral lung, vessel wall thickness was inversely correlated with FEV_1. Peinado et al. concluded that small pulmonary arteries of patients with mild COPD have endothelial dysfunction and intimal thickening (41, 42). In a recent paper, Santos et al. quantified the (immuno-)histochemical staining pattern of various extracellular matrix components including elastin, total collagen and proteoglycans with the same visual scoring method employed by us and, previously, by several other authors (23, 24, 43, 44). Santos et al. reported no differences in small pulmonary arteries between COPD patients and smoking non-COPD controls. They did, however, find a positive correlation between the amount of collagen deposition and intimal thickening (23). In analogy to the ECM deposition in the bronchial wall, damage to the endothelial lining can induce vascular remodeling, vascular smooth muscle proliferation, metaplasia of VSM to (myo-)fibroblasts, and increased synthesis and deposition of extracellular matrix proteins such as collagens and fibronectin (45). Our results support this hypothesis, which is likely also to contribute to vascular remodeling during the development of COPD.

Correlation analysis revealed a significant inverse correlation of FEV_1 values and total collagen and collagen I and III staining in the SEBM, fibronectin in intima of mucosal vessels and laminin expression in airway smooth muscle. These findings

are consistent with the hypothesis of the development of structural abnormalities in the bronchial airway wall and in the vessel walls in patients with COPD causing airways obstruction. The exact mechanism remains unknown.

Taken together, our results indicate that COPD is associated with increased deposition of ECM components in the bronchial airway wall, as part of the airway remodeling and contributing to airflow limitation. Blockade of pathways that are likely to be involved in structural and functional abnormalities should be considered in the development of therapeutic interventions aimed to prevent chronic airflow limitation in COPD.

Acknowledgements

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5.6 References

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Chapter 6

Regulation of ECM proteins by FGF-FGFR$_1$ system in cultured human airway smooth muscle cells

Adapted from:

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6.1 Summary

Altered extracellular matrix (ECM) deposition contributing to the airway wall remodeling is an important feature of asthma and chronic obstructive pulmonary disease (COPD) of which the molecular mechanisms are poorly understood. We examined the mRNA expression of ECM proteins, like collagen I, III and fibronectin in cultured human airway smooth muscle (ASM) cells stimulated with 10.0 ng/ml fibroblast growth factor 1 (FGF-1) and/or FGF-2 or 5.0 ng/ml of transforming growth factor β_1 (TGF-β_1) for 1, 2, 4, 8, 16, 24 and 48h. Densitometric analysis of Northern blots showed increased mRNA expression of collagen I and III in ASM cells stimulated for 24h with TGF-β_1 or FGF-1, whereas the levels for these mRNAs did not change in FGF-2 stimulated cells. ASM cells constitutively expressed fibronectin mRNA, which remained unaltered after each stimulus. TGF-β_1 did not induce cell proliferation as determined by ^{3}H-thymidine incorporation assay and cell counts, whereas FGF-1 ($P<0.05$) and FGF-2 ($P<0.001$) previously induced cell proliferation. Cellular hypertrophy assessed by total protein over DNA ratio in ASM cells remained unaffected. Increased levels of TGF-β_1 were observed in the conditioned medium of FGF-2 but not FGF-1 stimulated ASM cells with a maximum of 209±9.5 pg/ml after 2-4h. We conclude that TGF-β_1 and FGF-1 stimulate mRNA expression of collagen I and III in ASM cells, suggesting their role in the deposition of extracellular matrix proteins by ASM cells in the airways of patients with chronic lung diseases such as asthma and COPD.

6.2 Introduction

Chronic airway disorders like asthma and chronic obstructive pulmonary disease (COPD) are a global health problem with increasing morbidity and mortality (1). One of the key pathological features of these diseases is thickening of airway walls, which is thought to be a result of a chronic inflammatory process, in which inflammatory cells such as granulocytes, macrophages and T-lymphocytes play a role (2). In addition, ASM cell hypertrophy and increased deposition of extracellular matrix proteins such as collagens, elastin, laminin and proteoglycans around the smooth muscle could also be involved in airway wall thickening (3, 4). The exact cellular and molecular mechanisms that are underlying these changes are poorly understood.

Recent studies found plasticity between distinct airway smooth muscle cell phenotypes in vivo, which could have functional as well as structural consequences for physiology in the airway wall (4). Hirst and colleagues showed that cell-matrix interactions, in addition to growth factors, could have important effects on ASM cell proliferation and phenotype (3, 4). These authors showed that ASM cells cultured on collagen I or fibronectin matrix have increased proliferation, whereas ASM cells grown on laminin proliferate more slowly yet express contractile proteins (3). Many growth factors and cytokines including fibroblast growth factor-1 (FGF-1), FGF-2 and transforming growth factor-β_1 (TGF-β_1) that are released from the airway wall have the potential to contribute to airway remodeling, revealed by enhanced proliferation and increased collagen expression (5).

TGF-β is a multifunctional protein that is involved in inflammation and connective tissue synthesis. Three different isoforms exists (TGF-β_1 to -β_3) of which the TGF-β_1 isoform is often the most potent and commonly found growth factor in fibrotic and regenerative tissues under different pathophysiological conditions (6). In fibroblasts, synthesis of ECM proteins including collagens, elastin, proteoglycans and fibronectin is induced by TGF-β_1 (7). The effects on ASM cells are less clear. Khalil and colleagues demonstrated that TGF-β_1 and TGF-β receptors are present on ASM cells and that the release of biological active TGF-β_1 under influence of plasmin can induce ASM cells to synthesize pro-collagen I in an autocrine manner (8-10).

A number of studies have demonstrated that members of the FGF family and their four high-affinity, transmembrane tyrosine-kinase receptors (FGFR1-4) can also contribute to tissue repair processes and fibrosis during chronic inflammation in chronic airway diseases (11, 12). Increased expression of FGF-1 and FGFR-1 has been shown during the development of lung fibrosis (13) and FGF-2 has been implicated in the pathogenesis of obliterative bronchiolitis in lung transplants (14). We have recently demonstrated that the expression of FGF-1, FGF-2 and FGFR-1 in ASM cells of bronchial airways of (ex-) smokers with COPD is increased as compared to non-COPD subjects. In addition, in cultured human ASM cells cell proliferation is induced by FGF-1 and FGF-2 (15, 16).

In the present study we investigated the effects of TGF-β_1, FGF-1 and FGF-2 on the expression of ECM components including collagen I, III and fibronectin in human ASM cells. We show that TGF-β_1 and FGF-1 but not FGF-2 can induce the mRNA of subtypes pro-collagen I and III. We also demonstrate that FGF-2 stimulates ASM cells to release biologically active TGF-β_1, which could involve an additional autocrine mechanism. Taken together, these findings indicate that TGF-β_1 and FGF-1 stimulated ASM cells can increase their synthesis of extracellular matrix proteins that may contribute to the pathogenesis of airway fibrosis and remodeling in chronic airway diseases like asthma or COPD.

6.3 Materials & methods

Human airway smooth muscle cell isolation and culture

Human airway smooth muscle cells were isolated and cultured as we described previously (17, 18). Briefly, bronchial smooth muscle was dissected from a fresh macroscopically normal lobar or main bronchus obtained from patients who underwent surgery for lung cancer. After removal of the epithelium, parts of smooth muscle were dissected free of adherent connective and parenchymal tissue under aseptic conditions. Smooth muscle pieces were incubated in Hank's balanced salt solution (HBSS; Life Technologies BV, Breda, The Netherlands) containing bovine serum albumin (BSA, 10 mg/ml), collagenase (type XI, 1 mg/ml) and elastase (3.3 U/ml; Sigma BV, Zwijndrecht, The Netherlands) at 37°C in a humidified incubator containing 5% CO_2/95% air. After enzymatic digestion, the cell suspension was centrifuged and the pellet was washed in Dulbecco's modified Eagle's medium (DMEM), (Life Technologies BV, Breda, The Netherlands) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), (Bio-Whithaker BV, Verviers, Belgium) supplemented with sodium pyruvate (1 mM), nonessential amino acid mixture (1:100), gentamicin (45 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (1.5 µg/ml), (Life Technologies BV, Breda, The Netherlands).

Cells were subsequently seeded at $2x10^5$ cells per 35 mm dish and maintained in culture by replacing the medium every 72 h. After 10-14 days in culture, ASM cells grew to confluence and were then detached by trypsinization (0.5% trypsin; 0.02% EDTA; Life Technologies BV, Breda, The Netherlands) and subcultured into 25 cm^2 tissue culture flasks. Cells were further subcultured in 75 cm^2 tissue culture

flasks. Immunocytochemical staining of confluent serum-deprived primary cultures of human ASM cells, using monoclonal antibodies to smooth muscle α-actin and smooth muscle-myosin heavy chain (SM1 and SM2), (Sigma BV, Zwijndrecht, The Netherlands), (17, 18), demonstrated that the cultures were essentially free (>95%) of other contaminating cell types.

Growth factor stimulation

Human ASM Cells in 75 cm^2 tissue culture flasks in passage V-VI were washed twice in phosphate buffered saline (PBS) and treated with serum free DMEM containing 1 μM insulin, 5 μg/ml transferrin and 100 μM ascorbate (Sigma BV, Zwijndrecht, The Netherlands) for 72 h. Using flow cytometric analysis of human ASM cells stained with propidium iodide, we previously found that 72 h of serum deprivation resulted in approximately 85% of human ASM cells remaining in the G_0/G_1 phase (18).

ASM cells were stimulated with 5.0 ng/ml TGF-β1 (Promega, Madison, USA). Control incubations consisted of ASM cells that were incubated with FBS-free DMEM alone or DMEM containing FBS. Different ASM isolations (n=3) were in this case stimulated for 1, 2, 4, 8, 16, 24 and 48 hours. In a different set of experiments, human growth–arrested ASM cells were incubated with 10.0 ng/ml human recombinant FGF-1 (Promega, Madison, USA) or FGF-2 (Sigma-Aldrich, St. Louis, USA) in 10.0 ml FBS-free DMEM. Control incubations consisted of ASM cells that were incubated with FBS-free DMEM alone or DMEM containing FBS. Three different ASM isolations were stimulated for 1, 2, 4, 8, 24 and 48 hours.

Isolation of total cellular RNA and Northern blot analysis

Treated and untreated human ASM cells were washed in PBS and total RNA was extracted from the cells by the guanidium thiocyanate-phenol-chloroform method as previously described (17, 18). The RNA concentration was estimated by optical density measurements and a DNA/protein ratio of ≥ 1.8 was accepted. Samples of total RNA (10 μg) were denatured at 65°C in formaldehyde containing loading buffer and size fractionated on a 1% agarose gel containing 2.2 M formaldehyde. Ethidium bromide stained gels were photographed and RNA was transferred onto Hybond-N membrane (Amersham Nederland BV, 's-Hertogenbosch, The Netherlands) by the alkaline downward capillary transfer method also described earlier (17). The filters were air-dried

and UV cross-linked in a gene linker (Biorad Laboratories B.V., Veenendaal, The Netherlands). Blots were hybridized with radiolabeled cDNA probes against human mRNA for pro-alpha-1 type I collagen (3.4 kb fragment), pro-alpha-1 type III collagen (5.5 kb fragment) and fibronectin (7.7 kb fragment) or a reference house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1.2 kb). Filters were washed under stringent conditions and exposed to Kodak X-OMAT films at -80^0C. Hybridization signals were quantified by measuring the intensity of the bands with Molecular Analist (V1.5) image analysis software (Biorad Laboratories, Hercules, CA). The measurements of the intensity of the bands were corrected for background. The signal of all the investigated ECM molecules were expressed relative to corresponding GAPDH value and this ratio of the bands was expressed relative to the same ratio of control ASM cell (treated with serum-free medium). This relative optical density (OD) in stimulated cells, depicted as fold induction versus controls was expressed as mean ± SEM from three individual isolations and statistically analyzed.

Enzyme-linked immunosorbent assay for TGF-β_1

Conditioned media were collected from FGF-1 and FGF-2 treated human ASM cells, after which active TGF-β_1 levels were assessed using human TGF-β_1 specific enzyme-linked immunosorbent assay (ELISA) kit obtained from Promega (The Netherlands) according to supplier. In brief, Nunc Maxisorp 96 wells ELISA plates (Sanbio, The Netherlands) were coated with TGF-β_1 coating antibody in 0.025 M Sodium/bi-carbonate solution at 4^0C overnight. Next, the plates were incubated with blocking solution (Promega, the Netherlands) for 35 minutes at 37^0C. A standard curve using recombinant TGF-β_1 protein was first established and subsequently 0.1 ml of conditioned medium was used to assay for TGF-β_1. Subsequently the samples were incubated with biotinylated TGF-β_1 detecting antibody. After addition of the streptavidin-peroxidase conjugate, tetramethylbenzidine (TMB) was added and the absorbency of the resulting colored product was measured after 30 minutes using an automated spectrophotometer at 450 nm (Biorad Laboratories BV, Veenendaal, The Netherlands). The concentration of TGF-β_1 was expressed in pg/ml. The lower detection limit of the TGF-β_1 ELISA method was 15.6 pg of TGF-β_1/ml.

[^{3}H]-thymidine uptake

Human ASM cells were detached from culture flasks with trypsin and resuspended in DMEM containing 10 % FBS. Cultured cells from three different subjects were plated at a density 1x10^4 cells/well in 96-wells plates and cultured for approximately 7 days until semi-confluence. Human growth–arrested ASM cells (quadruple in 96 wells plates) were incubated with 0.1, 1.0, 3.0, 5.0 and 10 ng/ml human recombinant TGF-β1 (Promega, Madison, USA) or FGF-2 (Sigma-Aldrich, St. Louis, USA) in 100 µl FBS-free DMEM. Control incubations consisted of ASM cells, which were incubated with FBS-free DMEM alone, or DMEM containing FBS. Three identical plates with seeded cells of three different ASM isolations were stimulated for 8, 24 and 48 hours. Five hours prior to the end of the treatment 10 µl (1µCi/10 µl in HBSS) of [^{3}H]-labeled thymidine (Amersham, Roosendaal, the Netherlands) was added to the wells, at a final concentration of 1µCi/110 µl per well. The medium was removed after 8, 24 and 48 hours and the cells were washed twice with cold PBS. The cells were detached with 50 µl trypsin for 10 minutes after which 50 µl PBS was added. The plates were frozen overnight at –20^{0}C after which the cells were harvested on glass fiber filters using a Filtermate 196 cell harvester (Packard, Meridan, USA) and the activity was counted using a Microplate Scintillation ß-counter (Topcount, Packard, Meridan, USA). Measured radioactivity was expressed as counts per minute (CPM). The mean CPM of quadruple wells and subsequently three different cell isolations were expressed as ratio as compared to control cells in serum free medium (fold-induction).

Cell proliferation

In a parallel series of experiments, human growth–arrested ASM cells (quadruple in 24 wells plates) were incubated with either 0.1, 1.0, 3.0, 5.0 or 10 ng/ml human recombinant TGF-β1 (Promega, Madison, USA) in 500 µl FBS-free DMEM for 24 and 48 hours. Control incubations consisted of ASM cells, which were incubated with FBS-free DMEM alone, or DMEM containing FBS. Cells were processed for cell counting in the Casey®1 system (Schärfe system GmbH, Reutlingen, Germany). After stimulation the cells were trypsinized with 50 µl for 10 minutes. Cells in suspension were added to 10 ml of Casey®1 isotonic solution (6.38 g/l NaCl, 0.2 g/l Na-tetraborate, 1.0 g/l Boric acid and 0.2 g/l EDTA). Cell numbers were measured and analyzed using Casey®1 system software.

Total protein/DNA Ratio estimation

In parallel experiments ASM hypertrophy was assessed in response to FGF-1, FGF-2 and TGF-β_1 by calculating the total protein to total DNA ratio. Cells were seeded in 24 well plates and after serum starvation of 72 hours stimulated with 10 ng/ml FGF-1 and FGF-2, and with 5.0 ng/ml TGF-β_1 as described for the proliferation experiments. After stimulation the cells were washed with PBS and homogenated with 1.0 M NaOH. The total DNA content was determined fluorimetrically using DAPI as described earlier and the total protein content was measured using Bradford method (17, 18). Standard concentration curves were generated using haring sperm DNA and BSA, respectively.

Statistical Analysis

Data in the figures are given as mean ± SEM. Statistical analysis was performed by the *Bonferroni t test*. Significance was accepted at $P<0.05$.

6.4 Results

Expression of ECM genes after TGF-β_1 stimulation

Expression of ECM genes was examined in ASM cells stimulated with TGF-β_1 (5.0 ng/ml) at several time intervals. Representative Northern blots showing the expression pattern of collagens I and III and fibronectin are shown in Figure 6.1A. All ASM cells expressed mRNA for the investigated ECM genes. Densitometric analysis revealed that Collagen I and to a lesser extent subtype III were induced time-dependently by TGF-β_1 (Figure 6.1B and 6.1C, respectively). Collagen I mRNA induction was maximal and significantly upregulated as compared to control between 16 and 24h of stimulation, whereas collagen III mRNA was maximal between 24 and 48h of stimulation ($p<0.05$), (Figure 6.1B and 6.1C, respectively). Fibronectin mRNA expression remained unaltered after TGF-β_1 stimulation (Figure 6.1D).

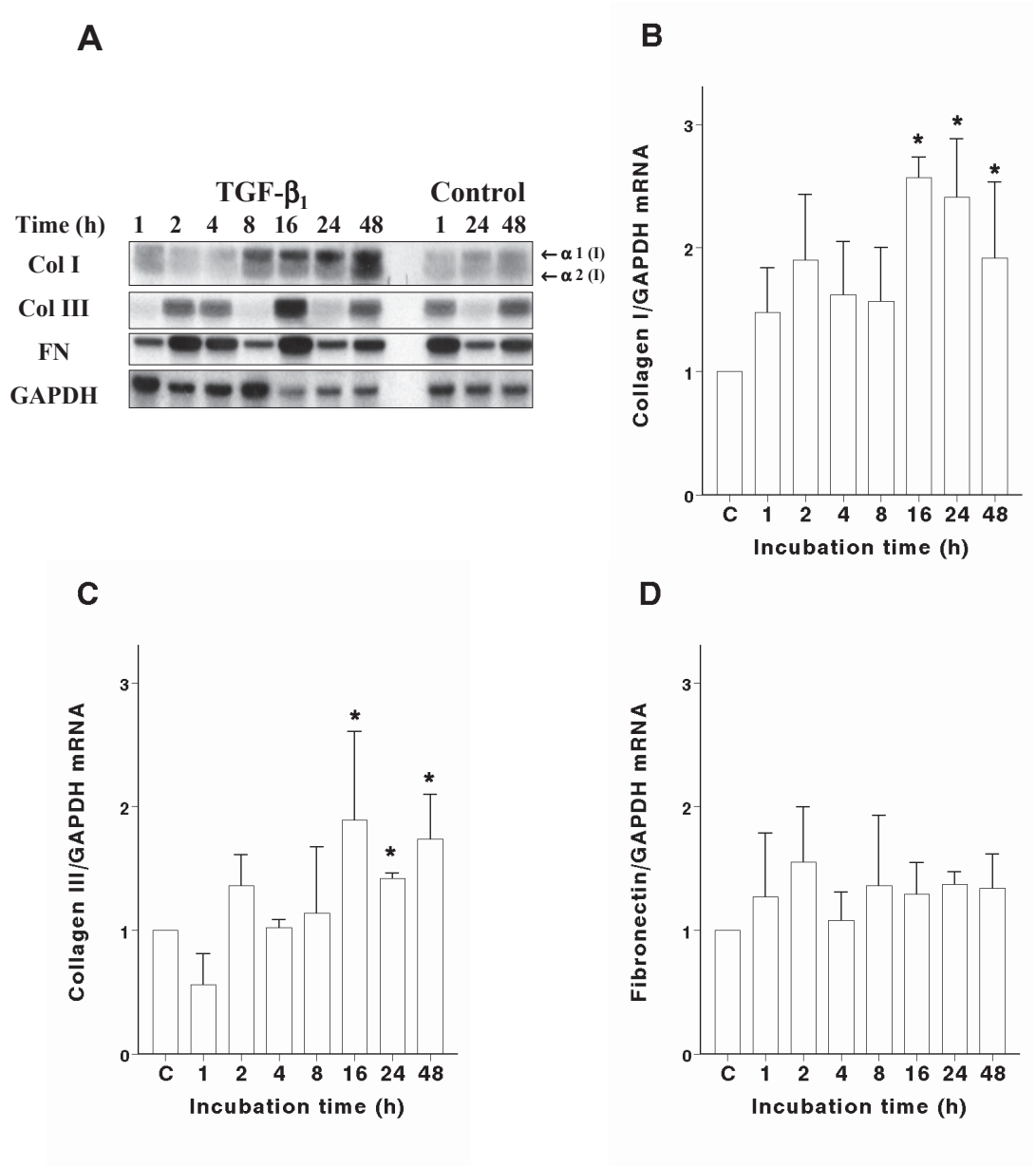

Figure 6.1 **Northern blot analysis of mRNA expression for extracellular matrix components collagen I, III and Fibronectin in human ASM cells in relation to TGF-β_1.** Serum-starved human ASM cells were incubated with or without 5.0 ng/ml TGF-β_1 for the times (h) indicated at the top of panel **A**. Total RNA hybridized with radio-labeled human probes against collagen I (Col I), Collagen III, (Col III), fibronectin (FN) and a house-hold gene GAPDH. Arrows on the right denote positions of pro-collagen α_1 (I) and α_2 (I) bands. Graphic representations of collagen I, III and fibronectin mRNA expression are depicted in panels **B**, **C** and **D**, respectively. Scanning densitometric values for ECM markers were normalized with respective GAPDH mRNA values. Values are means of the normalized signal ± SEM (n = 3) expressed as fold induction versus control (control value set at 1.0). * $P < 0.05$ versus control.

Expression of ECM genes after FGF-1 and FGF-2 stimulation

The expression of collagen I, III and fibronectin was also examined in ASM cells incubated with 10.0 ng/ml FGF-1 (Figure 6.2) or FGF-2 (Figure 6.3). We used 10 ng/ml FGF-1 or FGF-2, since this concentration had a maximal effect on proliferation of ASM cells in one of our previous studies. Representative examples of Northern blots for these ECM markers after ASM stimulation with FGF-1 and FGF-2 are shown in Figure 6.2A and 6.3A, respectively. Densitometric analysis of collagen I, III and Fibronectin mRNA expression, depicted as fold induction over control, are presented in Figure 6.2B, 6.2C and 6.2D for FGF-1 stimulated and in Figure 6.3B, 6.3C and 6.3D for FGF-2 stimulated ASM cells, respectively. Figure 6.2 and 6.3 illustrate that FGF-1 but not FGF-2 induced the expression of mRNA encoding both collagen I and III which were significantly increased between 24 and 48 hours of stimulation compared to untreated ASM cells ($p<0.05$). Fibronectin mRNA expression remained unaltered after stimulation with either FGF-1 or FGF-2 (Figure 6.2D and 6.3D).

Effects of FGF-1 and FGF-2 on the release of TGF-β_1 by ASM cells

The release of TGF-β_1 in the conditioned culture medium of ASM cells treated with FGF-1 or FGF-2 was measured to investigate an autocrine mechanism linking both growth factor systems. Table 6.1 shows a time dependent increase in the release of biologically active TGF-β_1 in the conditioned medium from FGF-2 stimulated ASM cells with a maximum at 2 hours (209±9.5 pg/ml) which declines after 4 hours (145±54 pg/ml) to 24 hours (47±13 pg/ml) compared to control (0 pg/ml). For FGF-1 we could only detect a TGF-β_1 signal (220 pg/ml at 4 hours) in one of three different conditioned culture media, which therefore did not reach significance compared to control. ASM cells treated with medium containing 10% FBS showed clear (exogenous) increase in biologically active TGF-β_1 at all time points which increased even further by applying 1.0 M HCl treatment to the samples, which caused the formation of active TGF-β_1 by releasing it from the latent form, in contrast to all experimental samples (data not shown).

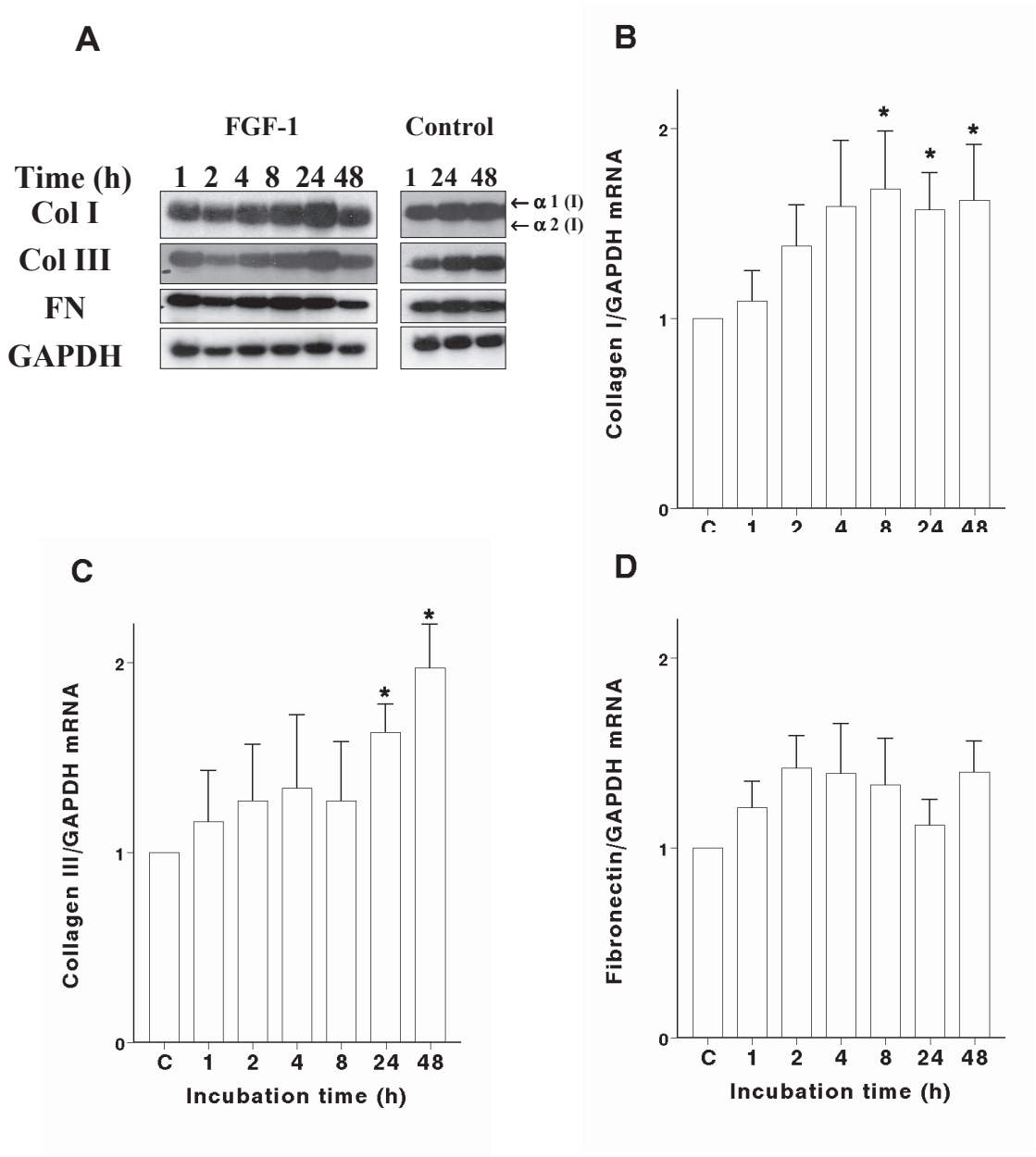

Figure 6.2 **Northern blot analysis of mRNA expression for extracellular matrix components collagen I, III and Fibronectin** in human ASM cells in relation to FGF-1 Serum-starved human ASM cells were incubated with or without 10.0 ng/ml and FGF-1 for the times (h) indicated at the top of panel **A**. Total RNA hybridized with radio-labeled human probes against collagen I (Col I), Collagen III, (Col III), fibronectin (FN) and a house-hold gene GAPDH. Arrows on the right denote positions of pro-collagen α_1 (I) and α_2 (I) bands. Graphic representations of collagen I, III and fibronectin mRNA expression are depicted in panels **B**, **C** and **D**, respectively. Scanning densitometric values for ECM markers were normalized with respective GAPDH mRNA values. Values are means of the normalized signal ± SEM (n = 3) expressed as fold induction versus control (control value set at 1.0). * $P < 0.05$ versus control.

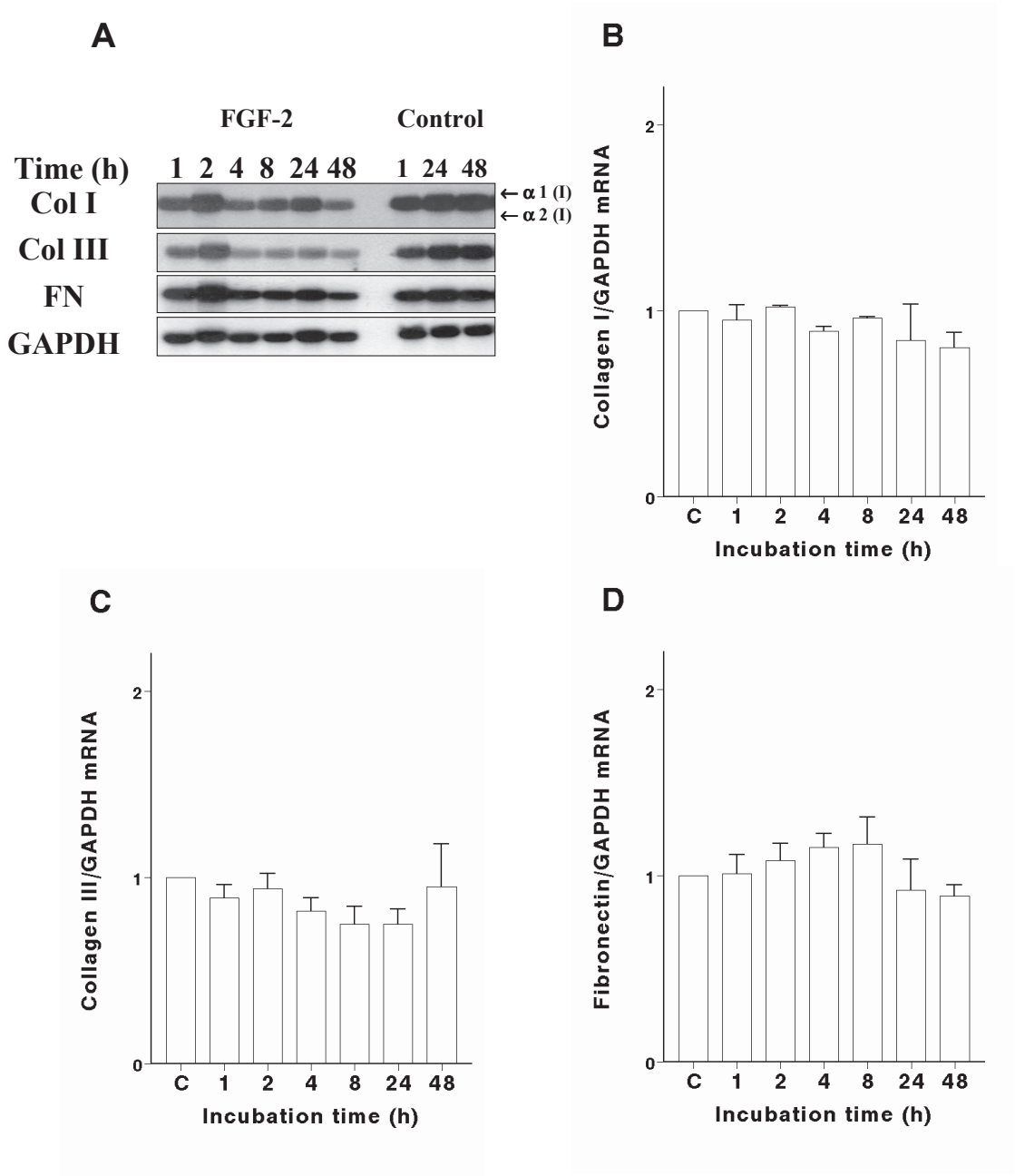

Figure 6.3 **Northern blot analysis of mRNA expression for extracellular matrix components collagen I, III and Fibronectin** in human ASM cells in relation to FGF-2 Serum-starved human ASM cells were incubated with or without 10.0 ng/ml and FGF-2 for the times (h) indicated at the top of panel **A**. Total RNA hybridized with radio-labeled human probes against collagen I (Col I), Collagen III, (Col III), fibronectin (FN) and a house-hold gene GAPDH. Arrows on the right denote positions of pro-collagen α_1 (I) and α_2 (I) bands. Graphic representations of collagen I, III and fibronectin mRNA expression are depicted in panels **B**, **C** and **D**, respectively. Scanning densitometric values for ECM markers were normalized with respective GAPDH mRNA values. Values are means of the normalized signal ± SEM (n = 3) expressed as fold induction versus control (control value set at 1.0). * $P < 0.05$ versus control.

Table 6.1 Effects of FGF-1 or FGF-2 on secretion of active TGF-β_1

	FGF-1	FGF-2
Active TGF-β_1 (pg/ml)	MEAN±SEM	MEAN±SEM
Control	0	0
2	0	209±9.5*
4	220±110	145±54*
24	9.8±9.8	47±13

Growth arrested ASM cells were stimulated with 10.0 ng/ml of FGF-1 and FGF-2 for up to 24 hours and the release of active TGF-β_1 was measured in the conditioned medium by ELISA. Values (mean ± SEM) are given in pg/ml and compared to control (untreated ASM cells) from three separate cultures. * P< 0.05 versus control.

Effects of TGF-β_1 on ASM cell proliferation and growth

Effects of TGF-β_1 on proliferation of ASM cells were investigated with [^{3}H]-thymidine incorporation and by determining changes in cell numbers in relation to untreated control ASM cells. Results for [^{3}H]-thymidine incorporation and the investigation of changes in cell numbers are summarized in Table 6.2 and 6.3, respectively. The [^{3}H]-thymidine incorporation after TGF-β_1 remained unchanged at all investigated time intervals (Table 6.2). Trends towards an increase at the later time intervals (24h and 48h) at higher concentrations of TGF-β_1 (5.0 and 10.0 ng/ml) could be observed but did not reach significance of $p<0.05$ (Table 6.2). In a separate set of experiments we investigated the changes in cell number in relation to TGF-β_1 stimulation expressed as fold induction over untreated ASM cells (Table 6.3).

Table 6.2 Fold changes in [^{3}H]-thymidine uptake of ASM cells in relation to TGF-β_1.

	8h	24h	48h
TGF-β_1 (ng/ml)	MEAN±SEM	MEAN±SEM	MEAN±SEM
Control	1	1	1
0.1	0.63±0.05	1.84±0.79	0.97±0.44
1	0.63±0.06	1.56±0.82	0.88±0.26
3	0.73±0.10	2.19±1.55	1.44±0.74
5	0.69±0.01*	2.56±1.41	1.77±1.05
10	0.79±0.05	1.77±1.06	2.85±1.70

Growth arrested ASM cells were stimulated with TGF-β (0.1 – 10.0 ng/ml) for up to 48 hours and the proliferation was examined by thymidine incorporation. Values are means of the normalized signal ± SEM (n = 3) expressed as fold induction versus control (control value set at 1.0). * P< 0.05 versus control.

The number of ASM cells after TGF-β_1 stimulation was increased at 24 hours of incubation at 3.0 or 10.0 ng/ml TGF-β_1 as compared to ASM cells which were (Table 6.3). At 48 h of incubation a relative increase in ASM cells treated with TGF-β_1 compared to their corresponding controls could not be observed. ASM cells treated with medium containing 10% FBS showed clear increase in [^{3}H]-thymidine incorporation and cell number at 24 and 48 h of incubation (data not shown).

Table 6.3 Fold changes in cell number of ASM cells in relation to TGF-β_1.

	24h	48h
TGF-β_1 (ng/ml)	MEAN±SEM	MEAN±SEM
Control	1	1
0.1	1.28±0.09	1.02±0.11
1	1.25±0.10	1.06±0.15
3	1.28±0.08*	1.11±0.17
5	1.51±0.25	1.11±0.13
10	1.43±0.12*	1.12±0.12

Growth arrested ASM cells were stimulated with TGF-β_1 (0.1 – 10.0 ng/ml) for 24 and 48 hours and the proliferation was examined by cell counting. Values are means of the normalized signal ± SEM (n = 3) expressed as fold induction versus control (control value set at 1.0). * P< 0.05 versus control.

In parallel experiments ASM hypertrophy was assessed in relation to FGF-1, FGF-2 and TGF-β_1 by calculating the total protein to total DNA ratio. Table 6.4 shows the effect of FGF-1, FGF-2 and TGF-β_1 on the protein/DNA ratio in human ASM cells. Of untreated cultured ASM cells the total protein/DNA ratio was approximately 120 when both measurement are normalized as ng/ml, which was similar as in one of our previous studies (17). No significant differences could be observed for any of the growth factors and at any of the time points investigated between treated and untreated ASM cells (Table 6.4).

Table 6.4 Protein/DNA ratio of ASM cells after TGF-β_1, FGF-1 or FGF-2 stimulation.

Incubation time (h)	Control	TGF-β_1	FGF-1	FGF-2
0	130±7.5			
24	125±7.1	122±11	142±4	144±6
48	124±8	130±12	133±9	132±4

Growth arrested ASM cells were stimulated with 10.0 ng/ml of FGF-1 and FGF-2 or 5.0 ng/ml TGF-β_1 for up to 48 hours and the protein/DNA ratio was measured. Values (mean ± SEM) are given as ratio of normalized to pg/ml protein and DNA concentration and compared to control (untreated ASM cells) from three separate cultures. * P< 0.05 versus control.

6.5 Discussion

In the present study we show that in human ASM cells pro-collagen α_1 (I) and α_2 (I) and collagen III mRNA expression are induced by TGF-β_1 and FGF-1 but not FGF-2. This effect is time dependent with a maximum at approximately 24 hours. We also show that the release of active TGF-β_1 was increase by FGF-2 treated ASM cells with a relatively fast time course and a maximum of 2-4 hours. Neither proliferation nor hypertrophy could by observed in TGF-β_1 incubated ASM cells.

TGF-β_1 is one of the most potent and well known inducers of extracellular matrix protein synthesis including collagens and fibronectin and could therefore be involved in tissue fibrosis in airways of patients with chronic respiratory disorders such as asthma and COPD (1-3, 8, 19-21). *In vitro* culture of human ASM cells experimentally injured could serve as a model for tissue damage and repair found *in vivo* situations (22). Coutts and colleagues showed recently that subconfluent or experimentally damaged human ASM cells release active TGF-β_1 increasing pro-collagen α_1 (I) and α_2 (I) mRNA expression (22). Our results of increased induction of mRNA expression of pro-collagen I and III in TGF-β_1 treated ASM cells confirm earlier findings that TGF-β_1 is able to induce the production of extracellular matrix proteins by ASM cells.

Fibroblast growth factor family members are also implicated in tissue remodeling in a wide variety of pathophysiological conditions including interstitial lung fibrosis. Barrios and coworkers showed FGF-1 and FGFR-1 expression in experimentally induced pulmonary fibrosis (13). Moreover, Becerril and colleagues showed that FGF-1 treatment of human lung fibroblasts resulted in down-regulation of collagen I synthesis and up-regulation of collagenases, a mechanism that may be protective against fibrosis (23). Furthermore, FGF-2 stimulation of vascular smooth muscle cells *in vitro*, in a model for vascular injury, has been shown to be associated with down-regulation of collagen type I and up-regulation of collagenase MMP-1 (24). Also in human dermal endothelial cells both FGF-1 and FGF-2 reduced the mRNA expression of collagen I and fibronectin, as compared to non-treated cells (25). Taken together, our findings of stable or slightly reduced mRNA expression of ECM components in ASM cells with FGF-2 treatment are in agreement with the result mentioned above. However, our results of increased mRNA expression of pro-

collagen α_1 (I) and α_2 (I) and of collagen III in FGF-1 stimulated ASM cells contradict previous findings. A recent study, however, indicated that FGF-1 but not FGF-2 could increase the expression of collagen I in human cultured skin epithelial cells as compared to non-treated cells (26). Possibly, cell-type differences could explain the discrepancies observed between several investigations.

In the present study we reported that active TGF-β_1 is released from FGF-2 and to a lesser extent FGF-1 stimulated ASM cells with a maximum at 2-4 hours of incubation. Since this induction is too rapid for de novo transcription and translation we hypothesize that this release originates from intracellular or cell-bound latent TGF-β_1 stores. Inactive TGF-β_1 is bound to latency-associated peptide (LAP), which together form latent TGF-β_1. Furthermore, latent TGF-β_1 is bound to latent binding protein-1 (LTP-1), which binds to the extracellular matrix. In this way, both the ECM and the latent TGF-β_1 complex serve as a reservoir for active TGF-β_1 (6). Although an exact mechanism remains unclear, a link between TGF-β_1 and fibroblast growth factors has been reported earlier (27, 28). Thannickal and colleagues showed in human lung fibroblasts that FGFR-1 (Flg) and FGFR-2 (Bek) were upregulated by TGF-β_1 incubation mediating enhanced mitogenic responses to FGFs. Secondly, FGF-2 release increased after TGF-β_1 stimulation, suggesting an autocrine loop for both factors (27, 28). A similar mechanism was indicated by Li et al., who showed that TGF-β_1 and FGF-1 could increase the release of FGF-2 from human cultured alveolar type II cells (29, 30). In human ASM cells, we demonstrate a reverse mechanism, the induction of TGF-β_1 by FGF-2, which has only been shown earlier to our knowledge in a cell line of glial origin as well as cultured neonatal astrocytes (31, 32). The role of FGF-2 stimulated TGF-β_1 induction in ASM cells is unclear. Taken together, we speculate that these findings could indicate a dual mechanism to regulate the synthesis of ECM.

We investigated the DNA synthesis, proliferation and total protein/DNA ratio in cultured human ASM cells stimulated with or without TGF-β_1 under serum-free conditions. Our results indicate that all three parameters mentioned above remained unaffected during the 48 hours of incubation TGF-β_1 in our study. Several reports commented on the proliferative effects of TGF-β_1 in cultured ASM cells indicating that TGF-β_1 has modulatory effects on proliferation with a condition-dependent nature (33-37). Black and colleagues found that 24 hours of incubation with TGF-β_1

decreased DNA synthesis, whereas 48 and also 72 hours increased DNA synthesis and proliferation in cultured bovine ASM cells (34). Interestingly, results from Okona-Mensah and coworkers indicated that TGF-β_1 inhibited 10% FBS induced DNA synthesis in sparsely seeded bovine ASM cells, whereas DNA synthesis was increased after 48 hours of TGF-β_1 treatment in the presence of only BSA in confluent grown cells (37). Cohen and coworkers found that thrombin induced DNA synthesis was inhibited by TGF-β_1 in human ASM cells and showed facilitating effects of TGF-β_1 on serum-induced proliferation and that TGF-β_1 incubation alone had no effect (35, 36). Our results are consistent with these latter findings. Apart from possible species differences, these observations suggest the existence of a dual pathway for TGF-β_1 modulated cell growth as seen for fibroblasts.

Taken together our results indicate that *in vitro* human ASM cells synthesize extracellular matrix components including collagen I, III and fibronectin, and in response to cytokines and growth factors such as TGF-β_1 and FGF-1 increase their collagen I and III expression and that interactions between TGF-β_1 and FGF-2 could modify these processes. These findings suggest that active TGF-β_1 release and the upregulated synthesis of extracellular matrix components by ASM cells resemble situations of fibrosis and airway remodeling during the pathogenesis of chronic airway diseases such as asthma or COPD. Structural alterations within the airway wall of asthma and COPD patients could lead to irreversible obstruction.

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Chapter 7

Summary and General Discussion

Summary & General Discussion

7.1 Outline of the Thesis

COPD is a chronic disorder of the lungs, with an abnormal inflammatory response of airways, parenchyma and vasculature in response to noxious particles and gases (1). The disease is becoming a major health problem with increasing trend of morbidity and mortality (1). COPD is a complex disease, which is influenced by genetic as well as environmental factors. Historically, the relation of α_1-antitrypsin deficiency and the development of COPD emphasized the role of genetics (2). Among factors like childhood respiratory infections, air pollution and occupational exposures, tobacco smoking is clearly the most important environmental trigger for COPD (2). Furthermore, strong relations of decline in lung function (as measured by FEV_1 % predicted) with the number of pack years as well as the beneficial effects of smoking cessation have been established (3). Yet of all smokers, only 10-20 percent actually develops COPD and in all subjects with diagnosed COPD only roughly 1% is associated with α_1-antitrypsin deficiency (3). The mechanisms determining these discrepancies as well as their underlying molecular mechanisms during the development and progression of the disease are not yet fully understood.

Tobacco induced injury is responsible for the process of chronic airway inflammation by an influx of inflammatory cells in the lumen and wall of bronchial and bronchiolar airways and as well as in the lung parenchyma. Structural abnormalities, in turn, will result in progressive airflow limitation and decreased gas exchange, in patients leading to breathlessness and eventually death. Yet, how smoke-induced injury can lead to the development of deregulated tissue repair with scar tissue formation is not completely understood.

7.2 Research Questions

We hypothesized that altered molecular events caused by differential expressed genes underlie the observed structural changes in COPD. Summarizing, therefore, the aims of studies presented in this thesis were:

- What characterize the structural alterations in the development of COPD in the peripheral as well as central vasculature and airways.
- What is the role of growth factors like FGF-1, FGF-2 and their receptor FGFR-1 as well as VEGF and its two receptors flt-1 and KDR/flk-1 in the development of vascular structural abnormalities in patients with COPD.
- What is the expression pattern of extracellular matrix (ECM) proteins such as collagens, laminins and fibronectin in the central and peripheral that could also contribute to airflow limitations in COPD.
- What is the effects of fibroblast growth factors and transforming growth factor-β_1 (TGF-β_1) on proliferation and production of ECM components by cultured ASM cells, as contributing cells to airway wall thickening in COPD.

7.3 Summary

Chapter 1 provides an overview of the clinical characteristics, pathogenesis and pathological changes in COPD. The inflammatory and structural abnormalities in COPD are described and the role of cytokines and growth factors in airway as well as vascular remodeling, pulmonary angiogenesis and the development of fibrosis in the peripheral and central airways are introduced. The aims of this thesis are outlined at the end of the chapter.

Chapter 2 focused on the vascular alterations in the peripheral lungs of COPD patients. We found structural abnormalities and increased protein expression of the fibroblast growth factor/receptor (FGF/FGFR) system in the peripheral pulmonary vasculature of COPD patients. COPD patients showed increased thickness of the pulmonary vessel walls in vessels with several sizes from 100 to 400 μm and above but not with vessels of smaller lumen diameter. Surprisingly, no significant differences were observed in the percent of smooth muscle content of the wall, as indicated by α-smooth muscle actin staining divided by vascular wall area. Interestingly, we observed that in COPD patients FGF-1 was significantly increased

in medial VSM cells of pulmonary vessels > 200 μm, whereas FGF-2 was more intense in endothelial and medial VSM cells of small caliber vessels (< 200 μm). Moreover, the expression of their receptor FGFR-1 was more pronounced on endothelial and medial VSM cells of each size categories in COPD patients. Furthermore, we observed an inverse correlation of FEV_1 with the medial VSM expression of both ligands and with vascular wall area. Therefore, the FGF/FGFR system could play an important role in the regulation of vascular remodeling, in COPD.

In ***chapter 3*** we described the pulmonary expression of vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1), which could also play a role in tissue remodeling and angiogenesis in COPD. We examined the immunohistochemical staining of VEGF, flt-1 and KDR/flk-1 in central as well as peripheral lung tissues obtained from (ex-) smokers with or without COPD. VEGF, flt-1 and KDR/flk-1 immunostaining was localized in vascular and airway smooth muscle (VSM and ASM) cells, bronchial, bronchiolar and alveolar epithelium and macrophages. Additionally, endothelial cells throughout the lungs abundantly expressed flt-1 and KDR/flk-1. Within the bronchial airways VEGF expression was enhanced in VSM cells of microvessels in the bronchial mucosa and submucosa as well as in ASM cells as compared to patients without COPD. VEGF expression was more intense in COPD in intimal and medial VSM of the peripheral pulmonary arteries associated with the bronchiolar airways and in small pulmonary vessels in the alveolar region as well. Moreover, KDR/flk-1 expression was enhanced in endothelial cells, intimal and medial VSM of the peripheral pulmonary arteries, whereas flt-1 expression in endothelial cells only. Furthermore, VEGF staining was significantly increased in bronchiolar, alveolar epithelium and bronchiolar macrophages as well as the flt-1 receptor in the bronchiolar epithelium. VEGF expression in bronchial microvessels in the mucosa, bronchial ASM cells and bronchiolar epithelium inversely correlated with FEV_1 values. Taken, together, these results implicate also VEGF and its receptors, flt-1 and KDR/flk-1 in peripheral vascular and airway remodeling processes in COPD.

In order to extrapolate these findings we investigated in ***chapter 4*** the role of FGF-1 and FGF-2 and their receptor FGFR-1 in the central bronchial airways. FGF-1, FGF-2 and FGFR-1 were quantified with digital image analysis and were localized in bronchial epithelium, airway and vascular smooth muscle (ASM and VSM). In COPD

as compared to non-COPD patients, elevated levels of FGF-1 and FGFR-1 were observed in bronchial epithelium and of FGFR-1 in only ASM. Interestingly, our results revealed increased expression of FGF-2 in COPD patients in the cytoplasm of the bronchial epithelium and nuclear localization in ASM cells. This latter observation could pinpoint towards an alternative functional mechanism for FGF-2. Moreover, we found a positive correlation of FGF-1 expression in the bronchial epithelium with packyears as well as inverse correlation of FEV_1/FVC with FGF-2 and FGFR-1 expression in ASM cells. Furthermore, in cultured human ASM cells, FGF-1 and/or FGF-2 induced cellular proliferation. Steady state mRNA levels of FGFR-1 were elevated in human ASM cells treated with either FGF-1 or FGF-2. Increased bronchial expression of fibroblast growth factors and their receptor in COPD cases, and the mitogenic response of human ASM cells to FGFs *in vitro,* suggest a potential role for FGF/FGFR-1 system in the remodeling of bronchial airways in COPD.

Furthermore***, Chapter 5*** showed that COPD is associated with increased deposition of extracellular matrix (ECM) molecules including collagens subtypes I, III, IV, fibronectin and laminin in the central airways, contributing to airway wall thickening. Staining for ECM components was observed surface epithelial basement membrane (SEBM) at sites of intact or damaged epithelium, interstitial space and vessels of lamina propria and adventitia of the bronchial airways. Total collagen was increased in the SEBM at sites of intact bronchial epithelium, but was not changed in the interstitial space and microvasculature of the lamina propria and adventitia of the airway in COPD as compared to non-COPD. Deposition of Collagen I and III, however, was enhanced in the SEBM both at damaged and intact epithelium, lamina propria and bronchial adventitia in COPD. Deposition of collagen IV was not different between the two groups, whereas expression of fibronectin was only increased in vessels of the lamina propria in COPD. Increased expression of laminin was observed in ASM and microvasculature in COPD as compared to non-COPD. FEV_1 values inversely correlated with collagen I and III in SEBM and lamina propria, respectively. When considering co-localization of total collagen with subtypes for collagen I, III and IV, we found a significant correlation between total collagen and collagen III in the SEBM at both damaged and intact epithelium but not between total collagen and collagen I or IV localization. We conclude that smokers with COPD

exhibit increased bronchial deposition of collagens, fibronectin and laminin and this could be involved in airway remodeling leading to airflow limitation.

In ***chapter 6*** we investigated whether the altered extracellular matrix (ECM) deposition in the central airways of COPD patients could be partly ASM cell derived. In this study, therefore, we examined the mRNA expression of ECM proteins such as collagen I, III and fibronectin in cultured human ASM cells stimulated with FGF-1, FGF-2 or TGF-β_1. Densitometric analysis of Northern blots showed increased mRNA expression of collagen I and III in ASM cells stimulated for 24h with TGF-β_1 or FGF-1, whereas the levels for these mRNAs did not change in FGF-2 stimulated cells. ASM cells constitutive expressed fibronectin mRNA, which remained unaltered after each of the stimuli. TGF-β_1 did not induce cell proliferation as determined by ^{3}H-thymidine incorporation assay and cell count, this in contrast to FGF-1 and FGF-2. Total protein over DNA ratio in ASM cells, as a measure for cellular hypertrophy remained unaffected by each stimulus. Interestingly, increased levels of TGF-β_1 were observed in the conditioned medium of FGF-2 but not FGF-1 stimulated ASM cells with a maximum after 2-4 hours of incubation. We conclude that TGF-β_1 and FGF-1 stimulate mRNA expression of collagen I and III in ASM cells. Taken together, induced cell proliferation by FGF-1 and FGF-2 and increased ECM synthesis by FGF-1 and TGF-β_1 in ASM cells *in vitro* implicate these growth factors in ASM cell accumulation by hypertrophy and/or hyperplasia during COPD.

7.4 Vascular alterations and the role of growth factors

Vascular abnormalities including pathological angiogenesis and vascular remodeling resulting from tobacco induced injury have been associated with the development of COPD (4-6). Early reports from Wright et al. described an increased wall area of small (< 500 μm) pulmonary vessel within the intima in mild to moderate COPD patients and additionally in the media in severe cases (4, 7). The wall thickening has been attributed to a chronic inflammatory process with ongoing fibrosis and an increased adventitial infiltration of inflammatory cells, predominantly $CD8^{+ve}$ T-lymphocytes (6, 8). The emergence of smooth muscle cells within the intima of small pulmonary arterial branches and the extension of medial vascular smooth muscle (VSM) distally into pulmonary arteries, arterioles and veins that are normally devoid of smooth muscle have also been described (9). We have looked at the vascular alterations in COPD and have shown vascular wall thickening in the peripheral pulmonary vessels of mild COPD patients compared to non-smoking controls (chapter 2).

Angiogenesis in COPD

Tobacco smoking imposes severe oxidative stress on the lungs directly via reactive oxygen species in the smoke as well as indirectly through activation of inflammatory cells leading to a repetitive cycle of oxidant stress and protease activation. Occluded capillaries and loss of the pulmonary vascular bed by emphysema has been suggested to lead to the formation of new vessels (angiogenesis) and an increased number of broncho-pulmonary arterial anastomoses (9). Hypoxia is an important trigger for angiogenesis in order to (re-)supply tissues with oxygen and detecting as well as responding to hypoxia are therefore of pathophysiological and clinical relevance (10). Sustained alveolar hypoxia can cause pulmonary vasoconstriction with pulmonary hypertension and pulmonary angiogenesis with the formation of collateral vessel sprouting and remodeling of existing vessels (9).

In COPD patients we observed increased expression of FGF-2 and receptor FGFR-1 in endothelial and VSM cells in many small calibre (50-200 mm) alveolar vessels (Chapter 2). Additionally, VEGF and its receptors, flt-1 and KDR/flk-1 were increased on these pulmonary vessels (Chapter 3). Angiogenic sprouting is a mechanism, in which VEGF and FGF-2, play an important role (11). It is assumed

that tobacco-induced tissue injury to the endothelium with consecutive alveolar hypoxia leads to a series of events initiating angiogenesis in COPD (12, 13). In brief, myo-fibroblasts or vascular smooth muscle cells get activated by hypoxia and expression of hypoxia inducible transcription factors is induced, resulting in VEGF secretion (10, 14, 15). Endothelial and VSM cells activation leads to destabilization of the vessels by the actions of angiopoietin 2 and tie-2 receptor (16-18). In addition, VEGF increases vascular permeability, thereby allowing extravasation of plasma proteins which lays down a provisional matrix for proliferation and migration of endothelial cells (18). The increase in vascular permeability and as well as additionally secretion of proteinases by endothelial and VSM cells lead to liberation and activation of growth factors such as VEGF and FGF-2 from the surrounding matrix with prolongation of endothelial cell initiated tube formation (16-18). FGF-2 and platelet-derived growth factor also affect angiogenesis by recruiting mesenchymal progenitor cells (pericytes) or (myo-)fibroblast and smooth muscle cells, whereas angiopoetin-1 and transforming growth factor–β_1 further stabilize the newly formed vessel (18). In COPD little is known about the exact role of angiogenesis, but the relevance of the blood vessels in COPD is emerging by recent observations, indicating that severe emphysema is associated with pulmonary endothelial cell apoptosis and increased levels of oxidative stress makers as well as decreased VEGF and type 2 receptor (KDR/flk-1) expression (19). Moreover, treatment with a blocker of VEGF type 2 receptor caused emphysema in experimental animals placed in hypoxic conditions (20). In contrast, we observed increased VEGF expression in pulmonary vessels in a patient group with mild to moderate disease. It is possible that the kind of patients is responsible for the observed differences, mild COPD subjects in our case versus solely emphysema patients in case of the study above. These discrepancies could also pinpoint towards different stages of development or severity of the disease. In mild to moderate COPD patients increased expression of VEGF and receptors may indicate an active and partly successful response to tobacco induced injury, whereas the decreased expression observed by Voelkel and coworkers may represent a failing response at the end stage of the disease.

Thus, the presence of VEGF and its receptors, especially KDR/flk-1, in the lungs are associated with both maintenance, survival and the protection against apoptosis of endothelial cells and the initiation of repair by angiogenesis in response to tissue injury. Although further studies are necessary to elucidate the contribution of

the formation of new vessels (angiogenesis) in COPD, our results suggest that VEGF and its receptors, flt-1 and KDR/flk-1 as well as FGF-2 and receptor FGFR-1 are important players in the peripheral lungs during the development of COPD (chapter 2 and 3).

Vascular remodeling in COPD

Many of the factors of normal vessel formation are also active during pathological vascular wall remodeling with deregulated repair as a consequence of either direct tobacco-induced injury, inflammation or increased shear stress in COPD (18). A pathological link has been establish between pulmonary hypertension and the development of vascular wall thickening and remodeling (13, 15, 21). Microvessels of the normal adult lungs contain a mixed population of partially muscular and muscular vessels, the latter consists of separated muscular segments where preexisting smooth muscle cells are defined by an internal and external elastic lamina (22).

The sources of the newly formed cells during vascular remodeling have been a key issue of investigation. Recent studies indicate that the existing VSM contribute only relatively little to the increase microvascular smooth muscle population as indicated by a low proliferation index (23, 24). Rather, vessel wall thickness increases by migration of interstitial fibroblast to the vessel wall and by cells derived from de-differentiated VSM or even endothelial cells. VSM and endothelial cell-derived VEGF, FGF-1 and FGF-2 stimulate fibroblast chemotaxis and proliferation (25-30). We have demonstrated increased expression of these ligands in our COPD patient group (chapters 2 and 3). Release of the mediators such as platelet-derived growth factor, and endothelin-1 may also contribute to chemotaxis and alignment of these cells, whereas transforming growth factor-β_1 induced the expression of α-SMA in endothelial and fibroblast, the early marker of smooth muscle phenotype differentiation (18). Furthermore, we have shown that the ratio in the amount of α-SMA positive staining versus vascular wall area remained constant in growing vascular walls, indicating an overall increase in all the individual cell types and extracellular matrix, rather than a shift towards a particular cell type (chapter 2). TGF-β_1 is a potent inducer of ECM proteins synthesis in fibroblast and vascular smooth muscle cells such as collagens which may be involved in vascular wall thickening in COPD as indicated by a correlation with the amount of total collagen deposition in the vascular wall (5, 31). In addition, recent observations link alveolar hypoxia and the

expression of hypoxia-inducible transcription factors with the actions of VEGF and FGF-2 on endothelial, VSM cells and fibroblasts in the ongoing process of vascular remodeling. Hypoxia and endothelial injury induce the expression of VEGF in VSM cells as well as VEGF and KDR/flk-1 in endothelial cells, whereas the expression and release of FGF-2 can be upregulated in endothelial cells by increased shear stress (10, 14, 17, 32, 33). The release of these growth factors leads to increased proliferation of endothelial and VSM cells. Furthermore, Rose and colleagues showed that hypoxic fibroblast showed increased HIF-1α expression and VEGF release, inducing both fibroblast recruitment and proliferation, which in turn activated and increased the proliferation of VSM cells (15). In addition, growth factors such as VEGF, FGF-2 and PDGF and TGF-β released from macrophages and mast cells upon hypoxia near sites of vascular lesions may contribute the vascular remodeling (27, 34, 35). Moreover, a shift in HSPG-side chain, which is acting as the potent co-receptor for the FGFR-1, leads to a remarkably enhanced responsiveness of FGF-2 on endothelial cells under influence of HIF-1α during hypoxia (36). The cellular interactions within the vascular wall and some of most the important mediators during vascular remodeling in COPD are summarized in Figure 7.1 (chapter 2 and 3).

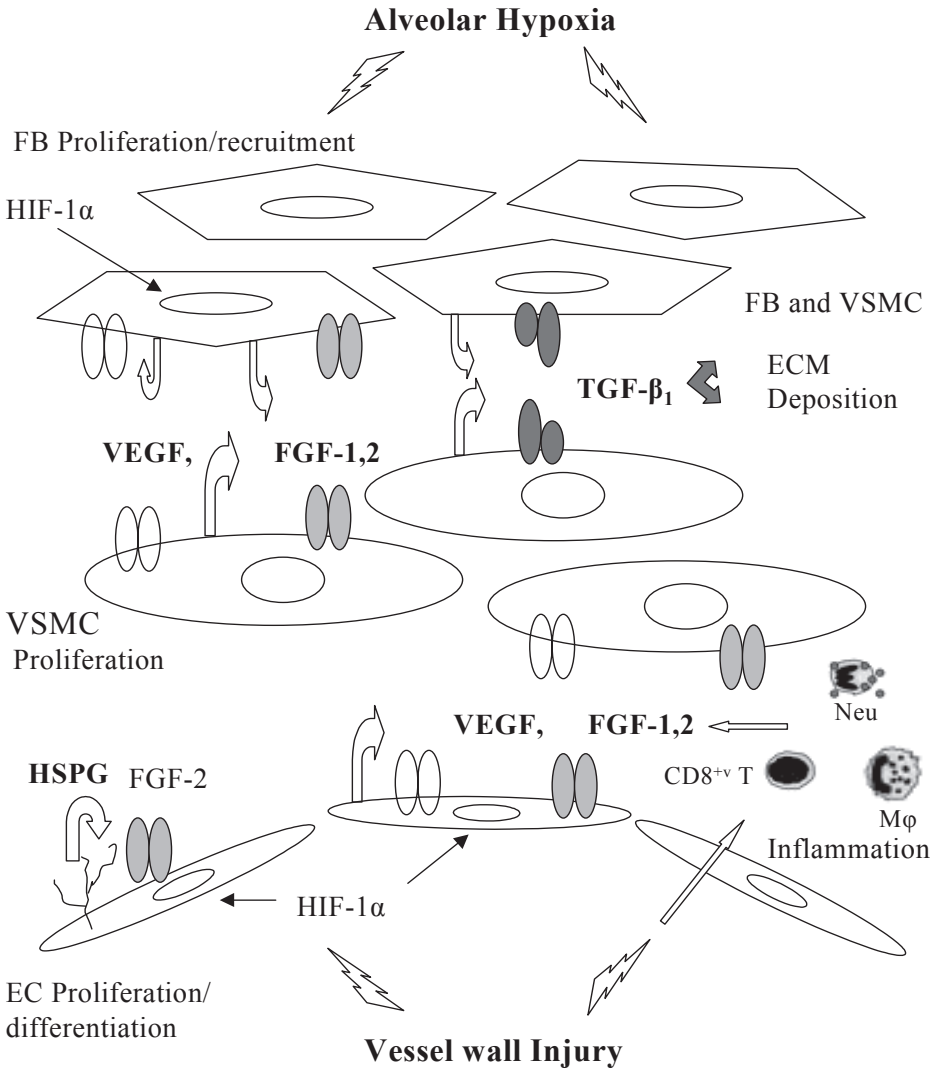

Figure 7.1 **Proposed mechanism of vascular wall thickening in COPD.** Cigarette smoking imposes severe stress on the lungs both directly, via the toxic agents and reactive oxygen species in the smoke and indirectly through the activation of inflammatory cells, predominantly neutrophils (Neu), macrophages (Mφ) and T-lymphocytes ($CD8^{+ve}$ T), causing tissue injury, that in turn leads to alveolar hypoxia. Moreover, pulmonary hypertension that is associated with COPD could lead to additional vessel injury via increased shear stress. Growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs) and transforming growth factor β_1 (TGF-β_1) released from inflammatory cells near sites of vascular lesions may contribute the vascular remodeling. Hypoxic fibroblast (FB) show increased hypoxia inducible factor (HIF) 1α expression and VEGF release, inducing both recruitment and proliferation of interstitial fibroblasts, and in turn proliferation of vascular smooth muscle cells (VSMC). Hypoxia and endothelial injury cause release of VEGF and FGF-2 from endothelial cells leading to increased proliferation of endothelial and VSM cells. During hypoxia endothelial cells show a HIF-1α dependent expression of heparan sulphate proteoglycan (HSPG) side chains. HSPGs act as co-receptors for FGF-1 and FGF-2, leading to a remarkably enhanced responsiveness of FGF-2. TGF-β_1 is involved in extracellular matrix (ECM) deposition within the vascular wall by FB and VSMC, and could initiate differentiation of EC and FB to a smooth muscle phenotype as indicated by the induction of α-SMA expression in endothelial and fibroblast. Several growth factors could play an important role in peripheral vessel remodeling during the development of COPD. Summarising, the investigated growth factors could play an important role in the pathophysiological processes that are active in peripheral vessel remodeling during the development of COPD.

7.5 Airway wall remodeling and the role of growth factors

Changes to small airways and lung parenchyma

Although the investigations in the pulmonary vasculature have gained interest, the role of the changes within the airway wall and have been studied intensively during the last several decades. The conducting airways can be subdivided in central, bronchial airway as well as more peripheral or small airways. Airways with a diameter of 2 mm or less are conveniently considered as small airways (37, 38). Inflammation and structural alterations in the small airways as well as the lung parenchyma are considered as the most important contributors to the airflow limitation and the accelerated decline of FEV_1 in COPD (37, 38). Many studies, therefore, have focused on the pathological changes that take places within the airways < 2 mm in diameter and lung parenchyma (37, 39-42).

Early reports showed that the specific morphologic features separating smokers from non-smokers were increases in epithelial and goblet cell metaplasia, smooth muscle mass as well as inflammation in the walls of small bronchioles and that young non-symptomatic smokers displayed early signs of inflammatory reactions in bronchiolar airways and alveolar air spaces without any apparent structural abnormalities (41, 42). Later studies further specified this increased inflammatory cell influx in COPD patients as predominantly neutrophils, macrophages, mast cells and $CD8^{+ve}$ T-lymphocytes (43-46). Changes in the lung parenchyma also contribute to the disease. As a result of this smoke-induced ongoing inflammatory processes, the connective tissue of the lungs gets degraded by a relative excess of inflammatory-cell derived proteases and a relative depletion of anti-proteolytic defences, together referred to as the protease-antiprotease hypothesis (47).

In the light of these observations, definite progress has been made in what factors can cause damage to lung tissue. The current knowledge in the development of COPD is summarized Figure 7.2.

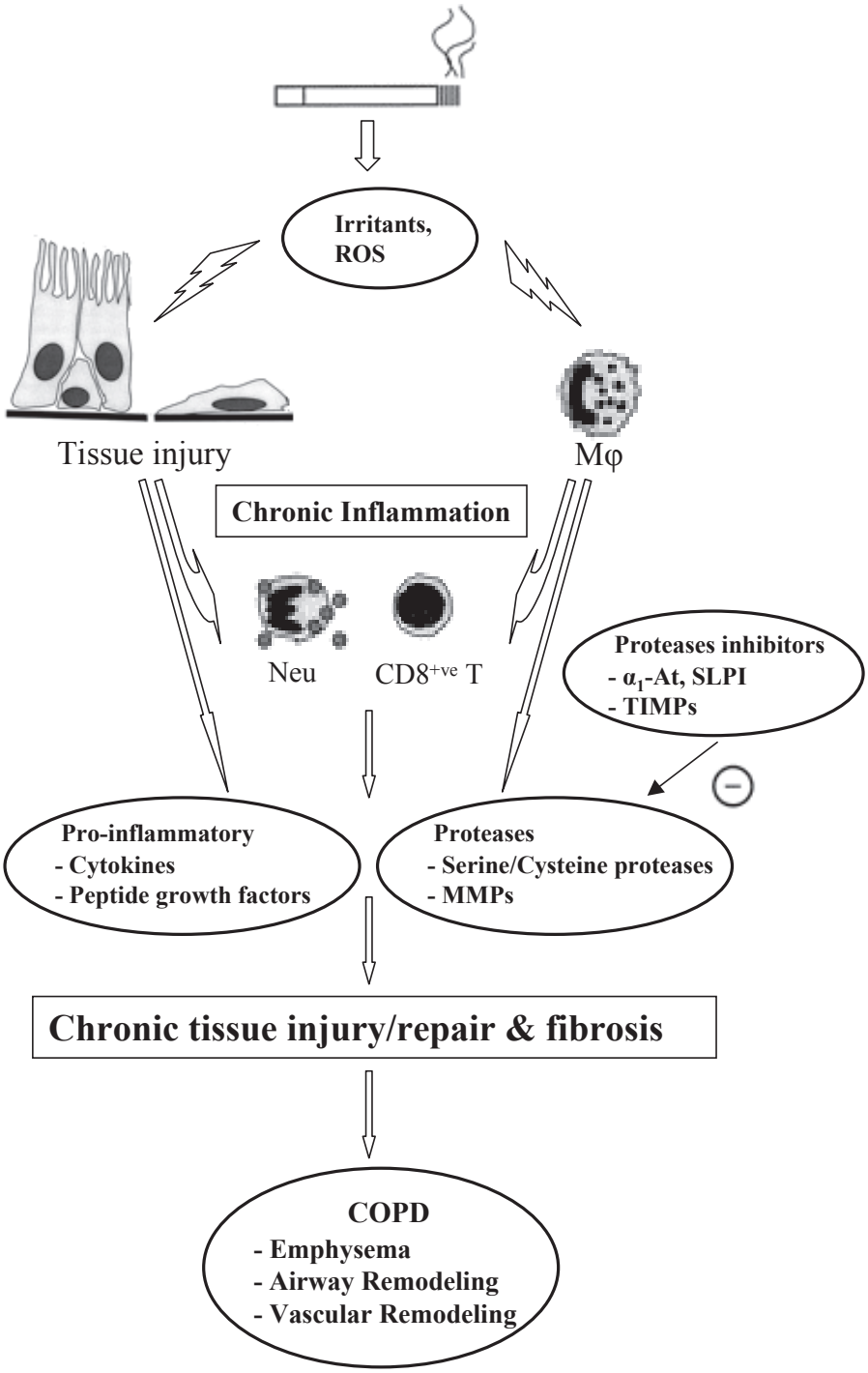

Figure 7.2 **Important mechanisms in the pathogenesis of COPD.** Chronic exposure to agents from tobacco smoke leads to tissue injury and chronic inflammation in the lung parenchyma, the small and large airways and vasculature with an influx of predominantly macrophages, neutrophils and $CD8^{+ve}$ lymphocytes. This leads to the release of many pro-inflammatory cytokines and growth factors and a misbalance in inflammatory and structural cell-derived proteases and their inhibitors (serine/cysteine proteases vs. α_1-antitrypsin, secretory leukoprotease inhibitor (SLPI), and matrix metalloproteinases (MMPs) vs. tissue inhibitors of MMPs, TIMPs). Excessive breakdown of elastin and collagens in the parenchyma with destruction of alveoli as well as increased deposition of extracellular matrix within the airways pulmonary vasculature with thickening and fibrosis contribute both to airflow limitation in COPD. Based on reference (48).

From recent human as well as animal studies it has become clear that COPD is characterized by breakdown of elastin but also breakdown and synthesis of collagen with scar formation by proteases including macrophage metalloelestase, neutrophil elastase and collagenases (47, 49, 50). Moreover, the main effector cells are probably resident macrophages as indicated by recent animals studies in which knockout mice for macrophage products such as macrophage metalloelastase which did not developed increased airspace sizes (emphysema) after chronic smoke exposure (51-53). Interestingly, knockout mice lacking neutrophil elastase were only 50-60% protected against smoke-induced lung injury and emphysema, which implies that neutrophils probably only partially contribute in this process (54). On the other hand, these results have to be taken with care because of possible differences between mice and men. Thus, several phenomena occurring in parallel may result in peripheral tissue destruction and remodeling in COPD. Little is known, however, about the exact role of peptide growth factors in the molecular mechanisms underlying these processes in the context of COPD.

Growth factors during tissue repair in COPD

Growth factors such as FGF-1, FGF-2, VEGF, PDGF, TGF-β_1 as well as many others produced and secreted by various cell types including inflammatory cells, bronchiolar and alveolar epithelial and airway smooth muscle cells or released from deposited extracellular matrix stores may contribute either adverse or protective to the process of airway remodeling (40, 46, 55). Chapter 2 focused on fibroblast growth factors in the peripheral lungs and we showed that FGF-1, FGF-2 as well as their receptor FGFR-1 were expressed by bronchiolar epithelial and airway smooth muscle cells, (myo-fibroblasts) and macrophages. Also VEGF and its receptor KDR/flk-1 and flt-1 (chapter 3) as well as TGF-β_1 and its receptor were found on bronchiolar and alveolar epithelial cells as well as airway smooth muscle cells (46). Moreover, we observed increased expression of VEGF on these cell types in COPD (chapter 3).

The role of growth factors in tissue remodeling is possibly ambiguous. FGF-1, FGF-2 as well as VEGF released from injured cells or deposited extracellular matrix stores are strong chemotactic agents for macrophages, mast cells and fibroblasts. Additionally, they prove to be potent mitogens for bronchiolar epithelial cells, (myo)-fibroblast and airway smooth muscle cells. FGF-2 and VEGF have been shown to be survival factors for epithelial cells as well. As indicated by a recent study from Pardo

and co-workers, FGF-2 prevented toxin-induced apoptosis in pneumocyte type II cells. Cell rescue relied on de novo protein synthesis of the anti-apoptotic proteins Bcl-X(L) and Bcl-2 within 4 h of FGF-2 treatment (56). Furthermore, the protective role of TGF-β_1 is emphasized by recent observation demonstrating that smoke extracts inhibited epithelial cells repair processes by interfering with the epithelial cell proliferation, motility and TGF-β_1 release (57). These data, suggest that epithelial cells present in the airways of smokers may be altered in their ability to support repair responses, which may contribute to architectural disruptions present in the airways in COPD, associated with cigarette smoking. Thus, fibroblast growth factors, TGF-β_1 and VEGF could play a role in effectively repairing damage to the lung epithelia and underlying connective tissues and protecting against further tobacco-induced tissue injury, in order to retain the normal architecture of the lungs.

Changes to large airways

Few studies of COPD have focused attention on larger airways of more than 2 mm in diameter. The characteristic changes in the central airways of smokers with established COPD include inflammatory cellular infiltration into the airway wall and mucous gland enlargement as well as changes in airway dimension in relation to lung function of patients with COPD (58-62). This last study showed that the wall area internal to the airway smooth muscle, the lamina propria, was significantly thickened over the entire range of cartilaginous airways, which was also associated with a reduction in FEV_1/FVC (62). Surprisingly, and in contrast to earlier reports from peripheral airways, alterations in large airway smooth muscle mass were not observed (62, 63). Therefore, those authors argued that their findings and those of others favor chronic inflammation with subepithelial fibrosis of the airways as a cause of the inner wall thickness. Bronchial microvessels in the lamina propria may contribute to the inner wall thickening by vascular wall remodeling or vascular edema, since the number of microvessels in the area 500 μm deep inside the airway wall appeared constant for patients with either COPD or chronic bronchitis as compared to smoking and also non-smoking controls (63).

Extracellular matrix and subepithelial fibrosis in COPD

Chapter 4 in this thesis described the role of fibroblast growth factors 1 and 2 as well as their receptor FGFR-1 in the bronchial airways during COPD. In COPD patients we found increased expression of FGF-1 in the bronchial epithelium, whereas FGF-2 was elevated in bronchial airway smooth muscle cells and FGFR-1 was more intense on both cell types. The central airways were also immunohistochemically positive for VEGF and its receptors KDR/flk-1 and Flt-1 and in COPD displayed increased expression for VEGF but not for its receptors in the bronchial epithelium, ASM cells, and the macrophages and microvessels in the lamina propria and adventitia of the bronchial airways (chapter 3).

Within the bronchial airways, collagen subtypes I and III, the most abundant ones, and fibronectin and laminin are found beneath the epithelial lining, throughout the interstitial spaces and in between most cells types and within the blood vessels of airway wall (64-66). Collagens and fibronectin are bound to cells through specific binding sites or receptors, the integrins, which are heterodimeric transmembrane receptors, consisting one α and β chain, which specially bind different ECM molecules (64, 65). Collagen IV and laminins are the main constituents of epithelial or endothelial basement membranes, which connects these cells, functioning as outward cellular lining of the airways or of blood vessels, with collagen subtypes I, III and VI from within the underlying interstitial spaces (67, 68).

In the light of damage and repair of the bronchial epithelium and the surface epithelial basement membrane (SEBM) as well as airway remodeling and fibrosis in underlying subepithelial regions including the lamina propria, airway smooth muscle, and adventitial layers, we also investigated the expression and deposition of various extracellular matrix molecules in the central airways of COPD patients (chapter 5). In chapter 5 we found within the surface epithelial basement membrane that the deposition of total collagen as well as subtypes collagen I, III and IV, fibronectin and laminin was increased at sites of epithelial denudation, irrespective of the disease state. Furthermore, COPD patients showed a significant elevation of the deposition of fibronectin, collagen I and III but not collagen IV or laminin as compared to non-COPD patients at the SEBM with or without the presence of epithelial damage (chapter 5). Moreover, in COPD patients collagen I and III but not fibronectin,

laminin and collagen IV were upregulated within the lamina propria and adventitia of the bronchial airways with accumulation of macrophages, fibroblast and α-SMA positive myo-fibroblasts (chapter 4 and 5). These results pinpoint towards ongoing bronchial subepithelial as well as adventitial fibrosis and airway remodeling in COPD. Within the bronchial airways, extracellular matrix is mainly produced by epithelial cells, (myo-)fibroblasts and airway smooth muscle cells. Bronchial epithelial cells and subepithelial fibroblast are rich sources of fibro-proliferative cytokines and growth factors as well as extracellular matrix products (69). TGF-β_1 is able to induce production of fibronectin and the release of VEGF in an autocrine manner in bronchial epithelial cells (70, 71). Interestingly, bronchial epithelial cell and fibroblast interactions with regard to extracellular matrix production were observed with cell culture. Conditioned media of bronchial epithelial cell were shown to induce macromolecule release accompanied by increased steady-state fibronectin and collagen I alpha mRNA levels (72). TGF-β_1 neutralizing antibody blocked this increase in extracellular matrix production, suggesting that TGF-β_1 produced by the epithelial cells may drive fibroblast matrix production (72). The increased deposition of collagen I and III within the interstitial matrix in the lamina propria and adventitial spaces could be produced by (myo-)fibroblast present in the bronchial airways (chapter 5).

The role of fibroblast growth factors on ECM molecule production appeared to be more variable among different cell types within the airways. In human epithelial cells FGF-1 has been shown to induce collagen I and III (73). Our results of increased FGF-1 expression together with its receptor FGFR-1 in the bronchial epithelium of COPD patients (chapter 4) could contribute to the elevated deposition of fibronectin, collagens I and III in the SEBM at sites with intact and especially at areas with denudation of the bronchial epithelium in COPD (chapter 5). It has been shown that TGF-β_1 is also able to induce FGF-2 from airway epithelial cells and that FGF-2 to induce collagen IV in human epithelial cells (73, 74). We found expression of FGF-2 expression on bronchial epithelial cells but observed no difference in FGF-2 expression as well as collagen IV deposition in COPD patients as compared to con-COPD patients (chapters 4 and 5).

Summarizing, the elevated expression of FGF-1 and its receptor FGFR-1, the increased expression of VEGF and the presence of KDR/flk-1 and flt-1 on bronchial epithelial cells and the increased deposition of ECM molecules in COPD, suggests a

mechanism of ongoing repair processes at sites of tobacco induced epithelial damage, triggering and perpetuating subepithelial fibrosis in COPD.

Airway smooth muscle, (myo-)fibroblast heterogeneity and the role in airway fibrosis

Evidence is emerging that (myo-)fibroblast and/or airway smooth muscle cells from diseases including asthma and idiopathic pulmonary fibrosis are phenotypically different compared to isolated cells from control patients (75-80). We found increased FGF-2 and FGFR-1 as well as VEGF in ASM cells of COPD patients, which could also contribute to smooth muscle mass increase and ECM deposition during airway remodeling in COPD (chapter 3 and 4). Moreover, in chapter 6 we showed that isolated ASM cells in vitro, treated with TGF-β_1 or FGF-1 but not FGF-2, displayed increased mRNA levels of pro-collagen subtypes III and I. Furthermore, in chapter 6 we described that active TGF-β_1 is released from FGF-2 and to a lesser extent FGF-1 stimulated ASM cells with a maximum at 2-4 hours of incubation. Moreover, FGF-2 and also FGF-1 but interestingly not TGF-β_1 induced proliferation of isolated ASM cells in vitro (chapters 4 and 6).

Normal mature ASM cells exist in vivo predominantly in a non-proliferative state with a fully differentiated contractile phenotype and expression of contractile makers (78, 81). The isolation and culturing *in vitro* on a serum-enriched medium with the exposure to many cytokines and growth factors causes the transition to a more proliferative phenotype, mimicking the events during chronic inflammation *in vivo* (78, 81). Serum deprivation restores the expression of most contractile markers. Intermediate forms may exist including a more "synthetic" phenotype with partly impaired proliferation, the synthesis of extracellular matrix components such as pro-collagen subtype I and the expression of some of the contractile elements like α-smooth muscle actin (α-SMA), together resembling a (myo-)fibroblast phenotype (77, 82-84). A recent study using isolated ASM cells demonstrated that ASM cells-derived TGF-β_1 localized extracellular and that plasmin regulated the secretion of a biologically active form of TGF-β_1 by ASM cells as well as the release of extracellular TGF-β_1. The biologically active TGF-β_1 induced ASM cells to synthesize collagen I in an autocrine as well as manner α-smooth muscle actin (α-SMA), (85).

During development of experimental lung fibrosis upregulation of FGF-1 expression is observed in fibroblast (86). As indicated by a recent follow-up study, however FGF-1 reduced the expression and synthesis of type I collagen and increased the collagenase protein expression were found in cultured human lung fibroblasts (87). Their findings demonstrated that FGF-1 might have a protective role in avoiding collagen accumulation during lung ECM remodeling. Also FGF-2 has been found to decrease mRNA expression and synthesis of the pro alpha-chains for types I and III collagen and to induce interstitial collagenase (MMP-1), which is required for degradation of collagen types I and III in vascular smooth muscle cells (88). Furthermore, FGF-2 completely disassembled the smooth muscle alpha-actin-containing stress fiber network and increased proliferation and migration of VSM cells (89).

Although an exact mechanism remains unclear, a link between TGF-β_1 induced ECM production and the role of FGFs increased proliferation has been proposed by recent investigations. Inactive TGF-β_1 is bound to latency-associated peptide (LAP) and this TGF-β_1 is bound to latent binding protein-1 (LTBP-1) and in turn to the extracellular matrix, servings as a reservoir for active TGF-β_1 (90). Release of bioactive TGF-β_1 by macrophages, ASM cells or from the ECM-bound reservoirs may occur by simultaneously released serine proteases of which plasmin is one of the most important (85, 91). Thannickal and colleagues showed in human lung fibroblasts that FGF-2 release increased after TGF-β_1 stimulation and that FGFR-1 (Flg) and FGFR-2 (Bek) were upregulated by TGF-β_1 incubation, mediating enhanced mitogenic responses to FGFs (92, 93). This suggests an autocrine loop for both factors.

In chapter 6 we show the opposite, the induction of TGF-β_1 by FGF-2 in human ASM cells, which has only been shown earlier to our knowledge in a cell line of glial origin and neonatal cultured astrocytes (94, 95). Since this induction was too rapid for de novo transcription and translation, we hypothesize that this release originates from intracellular or cell-bound latent TGF-β_1 stores. The role of FGF-2 stimulated TGF-β_1 induction in ASM cells is unclear. FGF-2, however, is known to induce plasminogen activator inhibitor-1 (PAI-1), blocking the cleavage from tissue–type and urokinase-type plasminogen activators (tPA and uPA), and thereby the formation of plasmin and thus of bioactive TGF-β_1 (96, 97). It could, therefore, be that

the decrease in bioactive TGF-β_1 is counteracted by its own induction by FGF-2. Taken together, these findings could pinpoint towards a dual mechanism to regulate pro-collagen I synthesis by actions of TGF-β_1 or FGF-2 on the level of plasmin. And in general, these growth factors could be involved in phenotypic switches between a proliferative/synthetic state versus a more contractile state.

Nuclear localization of FGF-2 in airway and vascular smooth muscle

In chapter 4 we show in COPD increased nuclear FGF-2, but not FGF-1, expression in airway smooth muscle cells by interactively counting of them using video image analysis. In chapters 2 and 4 we found that vascular smooth muscle cells also displayed this nuclear localization pattern. Currently, the role of FGF-2 in the nucleus has been partly clarified, as has been reviewed in two recent reviews (98, 99). The FGF-2 gene can produce at least five different isotypes: the conventional 18 kDa extracellular FGF-2, as well as four high molecular weight (HMW) forms (22, 22.5, 24 and 34 kDa). All four HMW isoforms, are able to translocate to the nucleus upon activation of different cells and in the nucleus, FGF-2 can act as modulator of ribosomal gene transcription (98, 99).

From several investigations it is becoming clear that the primary role of translocation of HMW FGF-2 isoforms to the nucleus is involved mechanisms of responding to cellular injury. Pro-inflammatory cytokines and growth factors such as interleukin-1 β (IL-1β), tumor necrosis factor α (TNF-α) and epidermal growth factor (EGF) were shown to selectively increase the expression of HMW-isoforms (22 and 24-kDa) but not of the conventional 18-kDa isoform, followed by nuclear translocation in cultured connective tissue cells (100). Also the FGF receptors can be translocated to the nucleus, as was evidenced by recent studies of Stachowiak and coworkers, showing increased expression and nuclear accumulation of basic fibroblast growth factor and the receptor FGFR-1 in primary cultured astrocytes following ischemic insults and in adrenal medulla cells after angiotensin II treatment (101-103). In fibroblast cell lines, overexpression of nuclear 24 kDa HMW FGF-2 is associated with increased resistance against toxic drugs and radiation induced DNA injury (104, 105). Additionally, cellular debris at sites of injury contains nucleic acid fragments released from dead cells and growth factors such as FGF-2. In viable but damaged surrounding cells, re-uptake followed by nuclear translocation of FGF-2 coupled to

DNA fragments can occur, which could be important events in early wound repair processes (106). Furthermore, Singh and colleagues also have shown that increased nuclear expression of 24 kDa HMW FGF-2 in vascular smooth muscle and endothelium precedes arterial enlargement in response to increased arterial blood flow *in vivo* (107).

Although the function of FGF-2 in the ASM cell nucleus in COPD patients remains unclear, from the pattern we observed we believe that the positive staining in the nuclei was not due to an artifact but representative of specific localization of the appropriate antigen by the antibody used. We hypothesize that pro-inflammatory cytokines that may be involved in perpetuation of chronic inflammation in COPD patients and the proliferation of airway smooth muscle (ASM) cells may rely on nuclear FGF-2 effects. Angiotensin II (Ang II), IL-1β and TNF-α, potent cytokines for a wide variety of cells including (myo-)fibroblasts and ASM cells, could be implicated in the expression and release of other fibro-proliferative messengers like TGF-β_1 and IL-6 by ASM cells (108-110). As indicated by recent studies, increased nuclear expression of 24 kDa HMW FGF-2 in ASM cells could be involved in the expression of cytokines like IL-6 by inducing gene transcription pathways (111-113). Taken together, these observations suggest that nuclear FGF-2 expression could be transcriptionally involved in a variety of compensatory mechanisms in response to cellular injury, which could indicate a novel FGF-2 and FGFR-1 signal transduction mechanism in COPD. The exact role of nuclear FGF-2 expression in COPD remains, however, to be elucidated.

7.6 Concluding remarks

Taken together, the investigated growth factors could play an important role in the pathophysiological processes that are active in airways as well as lung parenchyma during the development of COPD. The results presented in this thesis lead to the following conclusions:

- The protein expression of growth factors FGF-1, FGF-2 and their receptor FGFR-1 is increased in the pulmonary vasculature, which could be linked to the structural vascular abnormalities observed in COPD patients.
- The expression of the angiogenic growth factor VEGF-A and its receptors KDR/Flk-1 and Flt-1 are upregulated in the peripheral vasculature and airways of COPD patients, implicating VEGF-A and receptors in vascular and airway remodeling.
- COPD patients display more intense protein expression of FGF-1, FGF-2 as well as VEGF-A in the bronchial epithelium, airway smooth muscle cells, microvasculature and macrophages in the central airways, indicating their involvement in epithelial repair processes and the initiation and perpetuation central airway wall remodeling.
- The deposition of extracellular matrix components collagens I and III, fibronectin and laminin was increased in the bronchial airways of COPD patients as compared to non-COPD controls, contributing to bronchial airway wall thickening in COPD.
- ASM cells may contribute to bronchial wall thickening, indicated by their ability to produce the ECM markers collagen I, III and fibronectin in response to FGF-1 or TGF-β_1, as well as their proliferative response to FGF-1 and FGF-2 *in vitro*.

7.7 Implications for future research

The studies in this thesis indicate that growth factors (FGF-1, FGF-2, VEGF and TGF-β_1) expressed on various cell types and released from various sites in the lungs during chronic exposure to toxic agents from tobacco smoke, are important mediators in COPD. A rapidly growing number of cellular and molecular biomarkers with a large amount of possible interactions is implicated in the disease, reviewed by reference (48).

First of all, COPD is complex disease affecting all tree compartments of the lungs in a variable manner in individual patients, the lung parenchyma (emphysema), small airways (small airways disease) and the large airways (chronic bronchitis). The balance of inflammatory and structural cell-derived proteases as well as their inhibitors is also important in COPD (48). Excessive breakdown of elastin and collagens in the parenchyma with destruction of alveoli as well as increased deposition of extracellular matrix within the airways with thickening and fibrosis contribute both to airflow limitation in COPD. Thus, although evident progress has been made in the understanding of the disease, several important questions remain to be answered.

What is the individual contribution of different cells to the pathogenesis of COPD? In others words which of the already known cell types, intercellular mediators as well as intracellular messengers are involved in initiating and perpetuating the most important events of the three disease states in the lung parenchyma (emphysema), small airways (small airways disease) and the large airways (chronic bronchitis). Most likely several different mediators are involved in chronic inflammation, tissue damage and fibrosis. As reviewed recently, interesting targets for COPD treatment include anti-inflammatory drugs, antioxidants and anti-remodeling agents (48). However, new drugs for the treatment of COPD are needed and the identification of an association between peptide-growth factors such as FGF-1, FGF-2 and VEGF and the pathology of COPD could lead to new interventions either by promoting repair processes or preventing the formation of fibro-proliferative lesions.

Also of clinical importance for the progression of the disease, are mild and severe COPD differential stages of the same disease or totally different pathologies? The number of neutrophils, macrophages and $CD8^{+ve}$ T-lymphocytes in the peripheral

airways correlated with the severity of airflow limitation (44, 114). Furthermore, our observations that the cellular expression of several growth factors in the airways is correlated with the functional determinant of airflow limitation (FEV_1) emphasize their contribution to the disease. However, above observations do not rule out either possibility. If the progression from mild to severe COPD involves differential stages of the same disease, the question of reversibility of the disease is emphasized. Smoking cessation is obviously considered as beneficial, but further studies are necessary to investigate what the consequences are for the pathologic lesions such as the chronic inflammation and fibro-proliferative abnormalities in the airways of clinical COPD patients (115).

What is the role of the blood vessels in the pathogenesis of COPD and their possible contribution in the treatment of the disease? Although structural abnormalities in the blood vessels of COPD patients have been observed several decades ago, their importance has been re-emphasized by several recent studies. We observed that the vessels of COPD patients have increased expression of peptide-growth factors including FGF-1, FGF-2 and VEGF. Therefore, these peptide growth factors could be protective against tobacco-induced injury and may prove attractive therapeutic agents in the reversibility of the disease in the future.

The most intriguing question for the understanding of COPD is why only a minority of 10% of all smokers actually develops the disease, given the fact that the amount of exposure to tobacco smoke is comparable between cases and non-symptomatic smokers. Clearly, some people are more susceptible than others are, for the same amount tobacco smoked. Several genetic predispositions are identified, including associations between COPD and polymorphisms, in first of all α_1-antitrypsin, tumor necrosis factor-α and surfactant protein B genes (2). The associations above pinpoint towards differences in protection to alveolar destruction, in inflammatory mediator profile and in variations in lining fluid, respectively. The goal is to find which other heritable factors may contribute to the increased risk of development and progression of COPD. It would be interesting to investigate whether or not genetic polymorphisms can be found in genes that are involved in the initiation of repair processes and perpetuation towards pulmonary fibrosis, like peptide growth factors and their receptors.

7.8 References

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7.9 Samenvatting

De aandoening “Chronic Obstructive Pulmonary Disease” (COPD) is een verzameling van drie ziektebeelden die gekenmerkt worden door een chronische ontsteking met weefselschade in de longblaasjes (emfyseem), de kleinere luchtwegen (‘small airways disease’) en de hoofd bronchi (chronische bronchitis). Deze chronische ontsteking door de gehele longen is een gevolg van een langdurige blootstelling aan schadelijke gassen en deeltjes. Naast andere oorzaken, zoals luchtverontreiniging en blootstelling aan schadelijke stoffen tijdens werkzaamheden, is roken verreweg de meest belangrijke oorzaak. Het jarenlang excessieve gebruik van tabak leidt tot een beeld van progressieve achteruitgang van de longfunctie en het ontstaan van kortademigheid. Door de toegenomen tabaksconsumptie, voornamelijk gedurende het midden van de vorige eeuw, wordt COPD op dit moment een belangrijke oorzaak van morbiditeit en mortaliteit in de westerse wereld en neemt het aantal mensen dat aan de ziekte lijdt nog altijd toe. Er is een duidelijke relatie tussen de hoeveelheid gerookte tabak en afname van de longfunctie evenals een positief effect van tussentijds stoppen met roken. Echter, van alle chronische rokers ontwikkelt uiteindelijk slechts 10 procent daadwerkelijk klinisch aantoonbare COPD. Wat bepaalt welke individuen een verhoogde gevoeligheid vertonen voor chronische ontsteking en weefselschade, is een van de meest gestelde vragen aangaande de pathogenese van de ziekte.

Tijdens de voortdurende ontstekingsprocessen migreren immuuncellen onder invloed van onstekingsmediatoren en groeifactoren naar de plaats van de beschadiging. In rokers en in sterkere mate in COPD patiënten worden in het parenchym, de kleine en grote luchtwegen en in de bloedvaten verhoogde aantallen macrofagen, neutrofiele granulocyten en T-lymfocyten gevonden. Deze cellen scheidden bovendien extra beschadigende stoffen uit, waaronder reactieve zuurstof vormen en eiwitsplitsende enzymen. Hierdoor wordt het omliggende long weefsel herhaaldelijk beschadigd maar tevens gerepareerd. In bepaalde gevallen leidt een overvloed aan schade en een tekort aan herstel uiteindelijk tot irreversibele weefselvernietiging (emfyseem) evenals littekenvorming (weefsel herstructurering) in de longen. Deze structurele abnormaliteiten in de long resulteren in de progressieve longfunctieafname door middel van een verlaagde gasuitwisselingscapaciteit. De moleculaire aspecten van deze processen, echter, zijn onvoldoende bekend.

De doelstellingen van de in dit proefschrift beschreven studies waren daarom;

(I) het karakteriseren van structurele veranderingen in de centrale evenals perifere luchtwegen en het pulmonaire bloedvatstelsel,

(II) het identificeren welke specifieke groeifactoren mogelijk betrokken zijn bij deze weefsel herstelprocessen,

(III) in hoeverre veranderingen in afzetting van extracellulaire matrix macromoleculen inclusief collageen in de centrale luchtwegen de luchtweg obstructie beïnvloeden,

(IV) welke rol luchtweg gladde spiercellen spelen in de verdikking van de luchtwegen door productie van extracellulaire matrix moleculen en celvermeerdering onder invloed van specifieke groeifactoren.

Hoofdstuk 1 geeft een overzicht van de klinische aspecten, de huidige inzichten op het gebied van pathogenese en pathologie van COPD. De immunologische verschillen in de longen van niet-rokers, rokers en COPD patiënten evenals de belangrijke structurele veranderingen in COPD patiënten worden beschreven. De belangrijkste cytokinen en groeifactoren die betrokken zijn bij de ontwikkeling van luchtweg- en vasculaire herstructurering worden geïntroduceerd. De laatste paragraaf van het eerste hoofdstuk beschrijft de specifieke doelstellingen van dit proefschrift.

In ***Hoofdstuk 2*** stonden structurele verandering in de perifere bloedvaten centraal. We hebben met immunohistochemische technieken parenchymaal long weefsels van COPD patiënten en rokers zonder COPD onderzocht. Met beeldanalyse werden het oppervlak van de wanden en de diameter van de bloedvaten gemeten en vervolgens het oppervlak door de diameter gedeeld en alle bloedvaten gegroepeerd naar grootte. We vonden dat COPD patiënten vergeleken met controle patiënten een verhoogde wanddikte van de bloedvaten in verschillende groepen van 100 tot 400 μm in doorsnede en groter hadden, maar niet van kleinere afmetingen van 50 tot 100 μm in doorsnede. Om te onderzoeken of de hoeveelheid bloedvat gladde spiercel massa veranderd was in COPD patiënten ten opzichte van controles onderzochten we de bloedvaten op het gehalte kleuring voor de gladde spiercel marker, "α-smooth muscle actin", die niet verschillend bleek voor beide groepen.

Tevens werd in dit hoofdstuk de rol van fibroblast groeifactoren (FGF) 1 en 2 evenals hun receptor FGFR-1 in het perifere bloedvatstelsel in COPD beschreven.

Het FGF/FGFR systeem is van belang voor de groei en overleving van onder andere long fibroblasten en epitheel cellen maar ook van bloedvat gladde spiercellen en endotheelcellen. In COPD patiënten tonen we aan dat de FGF-1 eiwit expressie significant is verhoogd in bloedvat gladde spiercellen van pulmonaire bloedvaten die groter zijn dan 200 μm in doorsnede, terwijl FGF-2 juist in verhoogde mate gevonden wordt van bloedvaten van 50 tot 200 μm in doorsnede. Bovendien was hun receptor FGFR-1 in gladde spiercellen en endotheelcellen van vaten van beide categorieën significant verhoogd in COPD patiënten. Tenslotte, werd een negatieve correlatie van de belangrijkste longfunctie parameter "forced expiratory volume in one second (FEV_1)" met zowel de expressie van FGF-1 en FGF-2 in de bloedvaten als de mate van verdikking van de wand gevonden, wanneer beide patiënten groepen onderzocht werden. De beschreven groeifactoren zijn daarom mogelijk van belang in COPD patiënten bij de verdikking van de bloedvatwand door de actie van deze groeifactoren op de groei van bloedvat gladspiercellen en fibroblasten (vasculaire herstructurering).

In ***hoofdstuk 3*** werd de pulmonale expressie beschreven van een andere groeifactor die mogelijk betrokken is bij vasculair herstructurering en bloedvatvorming in de longen van COPD patiënten, "vascular endothelial growth factor (VEGF)" en zijn twee receptoren VEGFR-1 (flt-1) en VEGFR-2 (KDR/flk-1). Het proteïne expressie patroon van VEGF, flt-1 en KDR/flk-1 werd gekwantificeerd in perifeer longweefsel en evenals in de centraal bronchi van (ex-)rokers met en zonder COPD.

VEGF, flt-1 en KDR/flk-1 kwamen tot expressie in bloedvat en luchtweg gladde spiercellen, bronchiale, bronchiolaire en alveolaire epitheelcellen en macrofagen. Bovendien, brachten endotheelcellen door de gehele longen flt-1 and KDR/flk-1 in sterke mate tot expressie. In de bronchiale luchtwegen was VEGF expressie verhoogd in bloedvat gladde spiercellen van microbloedvaten in the bronchiale mucosa and submucosa lagen in de luchtweg gladde spier cellen vergeleken met patiënten zonder COPD. De expressie van beide receptoren KDR/flk-1 and Flt-1 was onveranderd tussen beide groepen in de bronchiale luchtwegen.

Ter hoogte van het longparenchym, was VEGF expressie toegenomen voor COPD patiënten in de intimale en mediale bloedvatgladde spiercellen van pulmonaire arteriën die geassocieerd zijn aan de bronchiolaire luchtwegen evenals in de kleinere parenchymale bloedvatvertakkingen. Bovendien, was in COPD de expressie van KDR/flk-1 verhoogd in endotheelcellen, intimale en mediale bloedvatgladde

spiercellen van pulmonaire arteriën evenals in de kleinere alveolaire bloedvatvertakkingen. Flt-1 expressie was voor COPD toegenomen in endotheelcellen van beide bovenstaande bloedvatcategorieën. VEGF kleuring was significant toegenomen in bronchiolaire en alveolaire epitheelcellen evenals bronchiolaire macrofagen, terwijl de flt-1 receptor alleen in het bronchiolaire epitheel was verhoogd. Tenslotte, werd een negatieve correlatie gevonden van de FEV_1 met de expressie van VEGF in zowel de bronchiale microbloedvaten in de mucosa, evenals in de bronchiale luchtweg gladde spiercellen en het bronchiolaire epitheel, wanneer de totale patiëntengroep onderzocht werd. Samengevat, wijzen deze resultaten uit dat VEGF en de twee receptoren, flt-1 en KDR/flk-1, betrokken zijn bij bloedvat- en luchtwegherstructurering in zowel de perifere long als in de centrale bronchustakken van COPD patiënten.

In ***Hoofdstuk 4*** beschrijven we de rol van FGF-1, FGF-2 en hun receptor FGFR-1 in de centrale, bronchiale luchtwegen. Het expressie patroon van FGF-1, FGF-2 en hun receptor FGFR-1 werd met behulp van digitale beeldanalyse gekwantificeerd. Beide groeifactoren en hun receptor kwamen tot expressie in het bronchiale epitheel, luchtweg gladde spiercellen en microbloedvaten in the bronchiale mucosa and submucosa. Significant verhoogde expressie vonden we in het bronchiale epitheel voor FGF-1, FGF-2 evenals FGFR-1 en in luchtweg- en bloedvat gladde spiercellen voor FGF-2 en FGFR-1. In gladde spiercellen was de expressie van FGF-2 nucleair, wat duidde op een alternatief, niet geheel opgehelderd, mechanisme van FGF-2 mogelijk door middel van regulatie van gentranscriptie via FGF-2. Bovendien vonden we een positieve correlatie van FGF-1 expressie in het bronchiale epitheel met de hoeveelheid gerookte tabak evenals een negatieve correlatie van FGF-2 en FGFR-1 expressie in luchtweg gladde spiercellen en de longfunctie parameter FEV_1/FVC, wanneer de totale patiëntengroep werd onderzocht.

Om meer inzicht te krijgen in het mechanisme van luchtwegverdikking door toename van luchtweg gladde spiercel massa, onderzochten we de proliferatie response van geïsoleerde humane gladde spiercellen op FGF-1 en FGF-2 stimulatie. We vonden dat na incubatie met beide groeifactoren, hoewel FGF-1 in mindere mate dan FGF-2, proliferatie van deze cellen geïnduceerd werd en dat de receptor FGFR-1 opgereguleerd werd. Samengevat, de verhoogde expressie van beide groeifactoren met hun receptor in de bronchiale luchtwegen in COPD patiënten en hun acties op

geïsoleerde humane luchtweg gladde spiercellen wijzen uit dat deze groeifactoren mogelijk een belangrijke rol spelen bij luchtweg herstructurering in COPD.

Hoofdstuk 5 beschrijft welke rol een mogelijk veranderde depositie van extracellulaire matrix eiwitten in de bronchiale luchtwegen speelt in luchtwegverdikking tijdens de ontwikkeling van COPD. In de bronchiale luchtwegen werd depositie van ECM eiwitten, zoals collageen I, III, IV, fibronectin en laminin, gevonden in de basaal membraan van het bronchiale epitheel, in de interstitieële ruimte en bloedvaten van de lamina propria en de adventitia voor zowel patiënten met en zonder COPD. COPD patiënten hadden een verhoogde depositie van totaal collageen in de subepitheliale basaal membraan, maar de depositie in de interstitieële ruimte en bloedvaten van de lamina propria en de adventitia bleek onveranderd te zijn. Wanneer we echter de hoeveelheid collageen I en III afzonderlijk bekeken, vonden we een verhoogde depositie in de subepitheliale basaal membraan zowel op plaatsen waar het epitheel intact was en een extra toename op plaatsen waar het epitheel beschadigd was en ook de depositie in de interstitieële ruimte en bloedvaten van de lamina propria en de adventitia was toegenomen ten opzichte van controles. De afzetting van collageen IV was op geen van de onderzochte plaatsen verschillend tussen beide groepen en fibronectin was in COPD alleen verhoogd in de microbloedvaten in de lamina propria. De depositie van laminin vervolgens was toegenomen voor COPD patiënten in luchtweg gladde spiercellen en ook in de microbloedvaten in de lamina propria. De belangrijkste bijdrage aan de toename van de ECM depositie in de subepitheliale basaal membraan werd gevormd door collageen III die een significante co-lokalisatie met de totaal collageen meting tentoonspreidde, in tegenstelling tot collageen I en IV. We concluderen dat COPD patiënten een verhoogde depositie van verschillende ECM markers hebben in de bronchiale luchtwegen dat mogelijk van belang is bij luchtwegherstructurering en het ontstaan van chronische obstructie.

In ***hoofdstuk 6*** onderzochten we of de verhoogde depositie in de luchtwegen van COPD patiënten, mogelijk gedeeltelijk afkomstig was van luchtweg gladde spiercellen. Naast bronchiale epitheelcellen en subepitheliale fibroblasten, vormen luchtweg gladde spiercellen tevens een belangrijke bron van extracellulaire matrix componenten. Daarvoor werden geïsoleerde humane luchtweg gladde spiercellen in

kweek gestimuleerd met FGF-1, FGF-2 en "transforming growth factor β_1 (TGF-β_1)" en werd gekeken naar de productie van mRNA voor ECM componenten collageen I, III en fibronectin door deze cellen. Met Northern blot analyse vonden we dat de mRNA productie van collageen I en III door luchtweg gladde spiercellen was verhoogd na 24 uur incubatie met FGF-1 en TGF-β_1 maar niet met FGF-2. De expressie fibronectin mRNA was onveranderd voor alle drie de onderzochte groeifactoren. Bovendien induceerde TGF-β_1 geen proliferatie van luchtweg gladde spiercellen, dit in tegenstelling tot FGF-1 en FGF-2 (Hoofdstuk 4). De ratio tussen totaal proteïne en DNA, die geldt als een maat voor cel hypertrofie wanneer deze toeneemt, bleef echter gelijk voor niet-gestimuleerde en gestimuleerde luchtweg gladde spiercellen met TGF-β_1, FGF-1 of FGF-2. Tenslotte, vonden we dat de secretie van actief TGF-β_1 toenam voor luchtweg gladde spiercellen die gestimuleerd waren met FGF-2 maar niet met FGF-1. Aangezien tevens is aangetoond dat TGF-β_1 kan leiden tot FGF-2 inductie, duidt dit samen op een mechanisme waarbij beide de secretie van de andere kunnen beïnvloeden. Samengevat, concluderen we dat luchtweg gladde spiercellen onder invloed van groeifactoren zoals TGF-β_1, FGF-1 of FGF-2 een bijdrage kunnen leveren aan luchtwegverdikking door een toename in gladde spiermassa en verhoogde depositie van extracellulaire matrix, die samen kunnen leiden tot luchtweg obstructie in COPD patiënten.

Hoofdstuk 7 geeft een overzicht van de belangrijkste bevindingen van dit proefschrift, beschrijft welke mogelijke mechanisme ten grondslag liggen aan vasculaire- en luchtwegherstructurering en wat de rol van de besproken groeifactoren in dit geheel is. Tevens wordt besproken wat de toekomstige implicaties hiervan zijn voor het onderzoek in het kader van weefselschade en herstelprocessen op het gebied van COPD.

I was told a million times
Of all the troubles in my way
How I had to keep on trying
Little better ev'ry day
But if I crossed a million rivers
And I rode a million miles
Then I'd still be where I started
Bread and butter for a smile.

"Keep Yourself Alive" (Queen)

Dankwoord

Waar zal ik beginnen! Alles is reeds verteld, bezongen, geschreven. The British Admiralty has reached saver shores, de Nijldelta is verlegd, de marathon van Tokio is reeds gelopen. Djengis is al jaren thuis en uitgeraasd! En, de bloemen voor de Sint Pieter zijn eerder al besteld en hebben de zegeningen ontvangen… Campagnes zijn gevoerd, en ook de mijne loopt nu op zijn einde. "My curtain calls" and the play reached the 50th over.

49.1

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49.2

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49.3

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functie bij Deltacell B.V. weer regelmatig in goede wetenschappelijke discussies tegenkom. Hiermee is de cirkel bijna rond, beste nieuwe collega's Menno, Jan, Pieter, Annelies en de heer J. Schram, dank jullie voor de steun met de laatste loodjes.

49.4

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49.5

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Curriculum Vitae

Andor Rogier Kranenburg werd op 6 december 1973 geboren te Gouda. Na het behalen van zijn VWO-B diploma aan de Dalton scholengemeenschap te Voorburg in 1992, startte hij met de opleiding Biomedische Wetenschappen aan de Rijksuniversiteit Leiden om deze in 1997 met het doctoraal examen af te ronden. Tijdens de doctoraalfase van deze studie werden wetenschappelijke stages gelopen bij de afdelingen Fysiologie en Fysiologische Fysica onder begeleiding van dr. L.J. Teppema en dr. A. Dahan, evenals Medische Biochemie onder supervisie van dr. J.A. Maassen, Rijksuniversiteit Leiden. De afstudeerstage, getiteld “Expressie van corticosteroid afhankelijke genen in de hippocampus die betrokken zijn bij neurodegeneratie na glutamaat receptor activatie” werd gevolgd bij dr. E. Vreugdenhil, Leiden Amsterdam Center for Drug Research (LACDR), vakgroep Medische Farmacologie, Rijksuniversiteit Leiden. Sinds juni 1998 is hij als assistent in opleiding werkzaam geweest aan een samenwerkingsproject, gefinancierd door het Nederlands Astma Fonds, tussen de afdelingen Farmacologie van de Erasmus Medical Center en Longziekten van het Leiden University Medical Center te Leiden. Het project getiteld "De rol van groeifactoren en extracellulaire matrix regulatoren in de luchtweg herstructurering bij chronic obstructive pulmonary disease (COPD)" werd begeleid door Dr. H.S. Sharma en Prof. dr. P.R. Saxena (Farmacologie, Erasmus MC) en Prof. dr. P.J. Sterk (LUMC). De bevindingen van dit werk zijn beschreven in dit proefschrift. In zijn huidige functie is hij werkzaam als scientist bij het respiratoir gerichte biotechnologische bedrijf “Deltacell B.V.” te Leiden in samenwerking met dr. Kielman (Deltacell B.V.) en de afdeling Longziekten van het LUMC.

List of Publications

Full papers

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3. Kranenburg AR, Willems-Widyastuti A, Mooi WJ, Saxena PR, Sterk PJ, de Boer WI and Sharma HS: Remodeling of Central Airways in Patients with Chronic Obstructive Pulmonary Disease: Role of FGF-1, FGF-2 and FGFR-1. *J Pathol* – In press

4. Kranenburg AR, de Boer WI, Alagappan VKT, Sterk PJ and Sharma HS: Enhanced Bronchial Expression of Vascular Endothelial Growth Factor and Receptors (flk-1 and flt-1) in Patients with Chronic Obstructive Pulmonary Disease. *Thorax*- In press

5. Sharma HS, Kranenburg AR, Alagappan VKT, Peters THF and Bogers AJJC: Angiogenesis and Vascular Remodeling in the Cardiopulmonary System. *Cell Biochem and Biophys* – In press.

6. Kranenburg AR, De Boer WI, Mooi WJ, Saxena PR, Sterk PJ, and Sharma HS. Chronic Obstructive Pulmonary Disease is Associated with Increased Bronchial Deposition of Extracellular Matrix Proteins, (*Eur Resp J*, In revision).

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1 Krishnan Parameswaran, Vijay K.T. Alagappan, Andor R. Kranenburg and Hari S. Sharma. 2004. Role of Extracellular Matrix and its Regulators in Airway Smooth Muscle Biology (submitted).

2 Kranenburg A. R., Willems-Widyastuti A., Alagappan V.K.T., Parmeswaran K., Sterk P. J., de Boer W. I. and Sharma H.S. 2004. Fibroblast growth factors differentially induce mRNA expression of collagens and fibronectin and secretion of transforming growth factor β_1 in cultured human airway smooth muscle cells. *Submitted.*

3 Kranenburg Andor R., Aarbiou Jamil, Alagappan Vijay K.T., de Boer Willem I., Sterk Peter J., Hiemstra Pieter H. and Sharma Hari S. 2003. Expression and autocrine mitogenic role of fibroblast growth factor-receptor system in bronchial epithelial cells, (in preparation).

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List of Abbreviations

Ang 1	angiopoietin 1
ASM	airway smooth muscle
BSA	bovine serum albumin
CD	cluster of differentiation
$CD8^{+ve}$ T	CD8 positive T-lymphocyte
cDNA	copy DNA
COPD	chronic obstructive pulmonary disease
DMEM	Dulbecco's modified Eagle's medium
EC	endothelial cell
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
Epi	epithelial cell
ET	endothelin
FBS	Fetal bovine serum
FEV_1	forced expiratory volume in one second
FGF	fibroblast growth factor
Flk-1	fetal liver kinase-1 (VEGF receptor 2)
Flt-1	fms-like tyrosine kinase (VEGF receptor 1)
FN	fibronectin
FVC	forced vital capacity
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HBSS	Hank's buffered salt solution
HIF	hypoxia inducible factor
HMW	high molecular weight
HSPG	heparan sulphate proteoglycan
ICAM	intercellular adhesion molecule
INF-γ	interferon gamma
Ig	immunoglobulin
IGF	insulin like growth factor
IL	interleukin
K_{co}	carbon mono-oxide constant

Mφ	macrophage
MAP	mitogen activated protein
MCP	monocyte chemotactic protein
MHC	myosin heavy chain
MMP	metalloproteinase
mRNA	messenger RNA
Neu	neutrophil
PAI	plasminogen activator inhitor
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
RAS	renin-angiotensin system
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
RV	residual volume
SEM	standard error of mean
SEBM	surface epithelial basement membrane
SMA	smooth muscle actin
SLPI	secretory leukoprotease inhibitor
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of metalloproteinases
TLC	total lung capacity
TNF-α	tumor necrosis factor-α
TPA/uPA	tissue-type or urokinase-type plasminogen activator
VSM	vascular smooth muscle
VEGF	vascular endothelial growth factor